ROLE OF INDIVIDUAL STIMULATOR CELLS AND MITOGENIC FACTORS IN HUMAN MIXED LYMPHOCYTE CULTURES

BY

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Dedicated to the memory of Thomas J. Ruiz

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COMMONLY USED ABBREVIATIONS

AEF	Allogeneic effect factor
°C	Degrees centigrade
Ci	Curie
CTL	Cytotoxic T cell
CTCl	Long term human T cell line
cpm	Counts per minute
ConA	Concanavalin A
DMSO	Dimethylsulfoxide
ESRD	End stage renal disease
ESA	Electronic sizing analysis
FACS	Fluorescence activated cell sorter
HC	Hydrocortisone
HLA	Major histocompatibility complex of man
H-2	Major histocompatibility complex of the mouse
IL1	Interleukin 1 or lymphocyte activating factor (LAF)
IL2	Interleukin 2 or T cell growth factor (TCGF)
I.U.	Individual units
L.D.	Lymphocyte-defined
LAF	Lymphocyte activating factor or Interleukin 1 (IL1)
Leu 2	Monoclonal antibody (Becton-Dickinson) which identifies human T cytotoxic/suppressor subset

Leu 3	Monoclonal antibody (Becton-Dickinson) which identifies human T helper subset
MNL	Mononuclear leukocytes (human peripheral blood)
MLC	Mixed lymphocyte culture (or reaction)
MF	Mitogenic factor
MLC-MF	Mixed lymphocyte culture-derived mitogenic factor
MHC	Major histocompatibility complex
N.D.	Not done
NA	Nonadherent human mononuclear leukocytes
NCS	Newborn calf serum
OKT4	Monoclonal antibody (Ortho) which defines human helper T cell subset
OKT8	Monoclonal antibody (Ortho) which defines human cytotoxic/suppressor T cell subset
PLT	Primed lymphocyte typing
PBS	Phosphate buffered saline
PHA-P	Phytohemagglutinin protein fraction
PWM	Pokeweed mitogen
SCID	Severe combined immunodeficiency
S.D.	Standard deviation
SD	Serologically defined
TCGF	T cell growth factor
x	Irradiated

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According to the clonal selection theory, lymphocytes proliferate and expand into specific sensitized clones when exposed to antigens. In the case of the MLC, the belief is that clonal proliferation of specific T cells exposed to allodeterminants on the stimulator cells determines the magnitude of the response, implying that the higher the number of HLA-D antigens mismatched between the responder and stimulator, the higher the MLC response.

However, it is well known that MLCs reacting to two HLA-D antigen differences are not always higher than one HLA-D difference, implying that other factors may play a role in an MLC.

The aim of this work was to explore the influence of those non-HLA-D factors in MLCs. One nongenetic factor identified as a major variable in MLCs was the intrinsic capacity of some individual cells to stimulate in MLC. Mononuclear leukocytes (MNL) from individuals were

found to behave as "high" or "low" stimulators to panels of responder cells in MLC. This capacity to stimulate was unrelated to HLA-D because cells from HLA-D identical individuals stimulated differently the same responders in the same experiment. The concentration of monocytes and B cells in a given population did not correlate with the capacity to stimulate in MLCs. Also, individuals identified as "high" stimulators induced MLC responses of equal or greater magnitude than pools of two or ten stimulators. One factor investigated as a potential modulator of lymphocyte proliferation was MLC-derived mitogenic factor (MLC-MF). These studies revealed that MLC-MF provides a powerful mitogenic signal to resting MNL, especially in the presence of irradiated, autologous monocytes. The action of MLC-MF is not genetically restricted or related to the presence of soluble alloantigens. Also, MLC-MF's signal to MNL was not found to be mediated through additional production of mitogenic substances. Column chromatography and absorption experiments suggested that MLC-MF and Interleukin 2 may be closely related. These studies have characterized stimulator cells and MLC-MF as two non-genetic factors that influence the extent of an MLC response. Thus, while only HLA-D antigens trigger the MLC reaction, it is a combination of several factors that determines its magnitude.

INTRODUCTION

The *in vivo* and *in vitro* immune reactions involving the major histocompatibility complex (MHC) done in the context of studying allograft rejection have now proved to be central in understanding the intrinsic capacity of an organism to develop any immune response. The mixed lymphocyte culture (MLC) has proved to be irreplaceable in examining genes in the MHC and also has provided a system of exploring the basis of lymphocyte activation by alloantigens. On the other hand, the MLC has some practical value in establishing genetic relationships among different individuals at a given MHC locus. Thus, the MLC has been and continues to be a unique probe to study fundamental immunological phenomena with a potential of practical application.

I. Cellular and Genetic Characteristics of the MLC

The MLC technique is a measurement of the recognition and resultant immune response by lymphocytes from an individual or animal when cocultured with lymphocytes from a genetically different member of the same species. The first description of the *in vitro* interaction between lymphocytes from unrelated individuals was made by Bain et al.(1) and Hirschhorn et al.(2) when they demonstrated that such mixed lymphocyte cultures developed responses comparable to mitogen stimulation of these cells. They observed in these two-way MLCs (where neither cell population is prevented from responding) the appearance of blast like cells which took up ^{3}H-thymidine in autoradiographic smears (a

measurement of DNA synthesis) and which underwent mitosis. The peak response in the cultures appeared 4-6 days after their initiation. Cultures containing lymphocytes from only one individual did not show any morphological change from resting lymphocytes and were not synthesizing DNA at any greater rate.

Subsequently, one-way MLCs (where only one cell population is proliferating) were developed which enabled the observation of a single individual's responsiveness to other "stimulator" lymphocytes. The latter were inhibited from proliferating by treatment with x-irradiation (3) or mitomycin C (4). The establishment of the microculture technique (5) enabled the use of smaller numbers of cells thus permitting analysis of a greater number of variables. The development of multisample harvesting machines (6) allowed for rapid and accurate determinations of the amount of cell-bound ^{3}H-thymidine that had been incorporated by the dividing lymphocytes. The use of ^{3}H-thymidine as a measurement of cell proliferation or MLC response has been the standard procedure used since its use was first proposed in 1964 (7).

Genetics of the MLC. Early studies on the mixed lymphocyte reaction (MLR) within families indicated that no reactions occurred between monozygotic twins but reactions were sometimes found between dizygotic twins, siblings and almost always between unrelated individuals (2,8). These initial indications of genetic influence upon the degree of MLC reactivity prompted the proposal that the MLC might be useful as an *in vitro* test of histocompatibility between individuals (8) and thus help select for compatible donors for transplantation.

In the mouse system, the use of H-2 congenic lines enabled Dutton (9) to determine that the major proliferative response in MLCs between strains occurred when the animals differed at the major histocompatibility complex (MHC). An analogous discovery was made in the human system in 1967 by Bach and Amos (10) who used siblings from seven large families and found correlation between stimulation in MLCs and leukocyte typings by sera from multiparous women (these sera typed a series of leukocyte antigens in a single system then known as Hu-1 and presently known as HLA). They proposed that Hu-1 contained the genetic loci which controlled the degree of reactivity in the MLC and that Hu-1 was probably the MHC in man. The same authors confirmed these findings (11) in a more in-depth analysis of the association of antigenic (serologically-defined HLA-A and -B loci) similarity and MLC stimulation, but found 3 individuals who although were serologically identical, mutually stimulated each other in MLC. Reactions in MLC between sero-identical individuals were also found by other investigators (12,13). By using a HLA recombinant family, MLC reactions between serologically identical siblings was shown to be closely associated with the HLA-B locus (14). These investigators proposed that a locus in or near HLA and distinct from those that were serologically identifiable could be the primary genetic stimulating locus in the MLC. The finding that only 10% of HLA-sero-identical unrelated individuals showed negative MLC responses (15,16) indicated that the relative stimulation in the MLC could not be predicted by HLA-A or HLA-B typing. When combined with the intrafamilial MLC data, these findings pointed to a locus in the HLA complex which strongly influenced MLC reactivity and was distinct from the serologically-defined (SD) antigens. The separate locus

which controlled the "MLC antigens" was named HLA-D by the Sixth International Histocompatibility Workshop in 1975 to distinguish it from the serologically-defined HLA-A,B and C antigens.

A similar situation exists in the mouse where the I region of the H-2 complex (analogous to HLA-D) which in general has a large influence on immune responsiveness and cellular interaction (17), also contains the major antigenic determinants recognized in the MLC (18,19,20).

Antibodies were discovered in multiparous sera absorbed with platelets (platelets carry HLA-A,B and C antigens) that reacted with B lymphocytes and which were later determined to be identifying determinants on molecules coded in or near the D region (21,22). These DR (D region related) antigens identified by these antibodies are the analogue of the Ia antigens in the mouse (23) and are found on B cells (24), macrophages (25), and activated T cells (26,27). The first indication of the association of DR antigens with the D region were the findings of inhibition of MLC reactions by DR antisera (22). Presently, there seems to be good correlation between the well defined DR antigens and the D antigens as defined by MLC typing (discussed below) (28). However, it seems that it is becoming difficult to maintain the parallelism between D and DR with the new specificities (28). Evidence that D and DR may be a separate locus includes the discovery of families with unusual D/DR haplotypes such as DW3/DRW2 (29), the report of a family which had a crossover between D and DR (30), and the finding of an antiserum which inhibited MLCs after absorption with a DR positive B cell line (31). However, as discussed by Bodmer (32), none of the apparent exceptions to the HLA D/DR association are absolute and all can still be explained on the basis of technical problems and incomplete

understanding of the genetics of this region of HLA. Recently, systems of DR-associated, serologically detected antigens, namely the MB antigens (33,34), MT antigens (35) and SB antigens (36) have been discovered. Their precise relationship to the D/DR region is presently unclear.

Attempts have been made to find methods of identification of the alleles of these "lymphocyte-defined" antigens of the HLA-D region. One approach has been through the use of homozygous typing cells (HTC) (37,38,39). Under the assumption that a lack of stimulation in MLC corresponds to HLA-D identity, cells were found which were homozygous at HLA-D, usually from offspring of first-cousin marriages. Panels of HTC are then used as stimulators in MLCs; if a responder shows minimal proliferation then this corresponds to compatibility between the responder and the HTC, whereas a high response indicates incompatibility. This can be exemplified in a theoretical MLC between individual A who has antigens 1 and 2 and individual B who is homozygous for antigen 2 (the HTC). In the MLC of B and Ax (x designating the irradiated or stimulator cell) there should be a reaction of responder B to antigen 1, since they share antigen 2. The reverse MLC (A plus Bx), however, should show little or no response since responder A recognizes no dissimilar HLA-D antigen. Thus person A, if his HLA-D phenotype was unknown, would be assumed to have antigen 2. Unfortunately, negative typing results are rarely this clear. With unrelated individuals, "zero reactions" (those close to the autologous control values) are usually not found (40) and compatibility at HLA-D is instead determined by consistently low versus high responses. In families, zero responses tend to occur more frequently (40). Various explanations have been put forth for this phenomenon. One is that determinants outside of HLA-D

capable of eliciting an MLC response, analogous to the *mls* locus in the mouse (41,42), would not be shared between the responder and stimulator cells. This would, in all likelihood, occur more frequently in MLCs between unrelated individuals than in intrafamilial MLCs, thus accounting for easier typings with families. In addition, in inbred mice, F_1 hybrid lymphocytes have been shown to proliferate to parental lymphocytes even though there is apparent genetic identity (43,44). This phenomenon has also been observed in inbred human families (21, 40). Other factors influencing the MLC could be the nonspecific proliferation of lymphocytes induced by blastogenic factors produced during the MLC (45) and varying levels of regulator or suppressor cells (46) in the responder population.

The other cellular method used for defining HLA-D specificities is the primed lymphocyte-defined typing or PLT (47,48) which was proposed as an alternate method of typing based on mouse PLT data. This method of typing is based on the concept that specific clones generated during the course of a MLC, when harvested several days after the maximal proliferative burst of the primary MLC, will show secondary response kinetics when restimulated with the original sensitizing cells or with cells sharing LD antigens of the original stimulator. Using this method of "positive typing," PLT cells can be generated against HTC (inducing clones specific for one HLA-D antigen) and then tested on panels of cells to determine what HLA-D specificity the cell possesses. HTC can also be used as the PLT cells. There are presently 12 HLA-D specificities as defined by negative and positive HTC typing methods presented at the Eighth International Histocompatibility Workshop. As with the negative typing, a major problem with PLT assays are the

unusually high "background" responses which are often seen and which inhibit easy interpretation of results. One reason for this may be the nature of the determinants which stimulate in the primary MLC as compared to determinants involved in stimulation of PLT cells in secondary MLC. As mentioned above, the PLT test yields reagents which recognize products encoded within the D region (47,48,49). However, it has been put forth by several groups that the HLA-D stimulating products in the primary MLC are distinct from the antigens recognized by MLC primed cells (50,51). They have proposed that DR antigens are the strongest stimuli recognized in the secondary response as demonstrated by a higher association with DR than D in PLT responses. Other unknown loci (PL) have also been implicated in PLT stimulation (52,53). This is further evidence that the serologically detected DR locus is separate from D. These differences in stimulating determinants recognized by memory cells may have an influence on interpreting PLT results. Another influence on the PLT response is that several clones are stimulated even against one haplotype difference, and differential proliferative capacity exists due to distinct determinants that are being recognized. One approach to reduce this variable has been through the cloning of PLT reagents (54). Their results indicate that a better discriminatory capacity exists with cloned cells. Attempts at treating the responder cells with nylon wool (55) have in one report aided in the interpretation of results.

Cells Involved in the MLC

Responder cells. The "thymus derived" or T lymphocyte appears to be the predominant cell which recognizes allodeterminants on the stimulator cell and which undergoes blastogenesis in the MLC. Abundant

evidence is available which shows that cells from neonatally thymectomized mice (56) rats (57) and chickens (58) do not respond well in MLCs. In the mouse system, treatment with anti-theta serum eliminates the response in MLC (59,60). Weber (61) used chromosomally marked T cells and B cells in chicken MLCs and found a selective proliferation of T cells. Cantor and Boyse (62,63) subsequently demonstrated that the T cells responding in MLC to I-region differences were of the Ly 1+ phenotype or helper T cell population. These Ly 1+ cells can also serve as helpers for the induction of cytotoxic T cells (Ly 2+, 3+ cells) during the course of a MLC (discussed below).

In the human MLC, T cells also constitute the major fractions of responding cells (64). Recently, monoclonal antibodies (the OKT series and Leu series) have been developed which detect human T cell subpopulations involved in the regulation of the immune response. The OKT4+ subset, which provides inducer (helper) function in T-T, T-B and T-macrophage interactions (65,66) is the predominant population which proliferates in the MLC (67). The other principal subset, OKT5+/8+ cells, also proliferates in MLC (67) but appears to contain the cytotoxic cells which develop following MLC and also suppressor cells (66). The Leu 3 monoclonal antibody basically correlates with OKT4 and the Leu 2 antibody with OKT5/8 (68). Differences in activities between the two series, such as the presence of a MLC induced Leu 3+ suppressor (69) may be due to varying methods of isolating the subsets, ("negative" selection with antibody and complement versus "positive" selection with the flourescence-activated cell sorter).

Stimulator Cells. The nature of the cell(s) responsible for stimulation in the MLC is not as clear and certainly more controversial than the nature of the responder cell. Several investigators have shown that both T and B lymphocytes, in the mouse and human system, stimulate well in MLC (64,70). Others have shown that B cells stimulate better than T cells (59,71-74), and one group has demonstrated that IgM-bearing B cells are the subpopulation responsible for B cell stimulation (75). Monocytes and macrophages have also been found to be effective stimulators in MLC (76,77,78) along with Ia-bearing, activated T cells (79), and dendritic cells (80). Not all Ia-bearing cells stimulate in MLC since B cells from leukemia patients which bear Ia antigens were shown to stimulate poorly in MLC (81).

As mentioned before, irradiated (3) and mitomycin-C (4) treated cells are able to stimulate and it has recently been reported that glutaraldehyde treated cells, while unable to proliferate, could still stimulate in MLC (82). Stimulator cells damaged by physical or other chemical agents have been described to lack any stimulatory ability in MLC (83,84), despite expressing "transplantation antigen" (85).

Development Of Primary And Secondary Cytotoxic Cells During The MLC. It has been known for sometime that allogeneic cell interaction results in the generation of cytotoxic T lymphocytes (CTL) which specifically lyse cellular targets, independent of antibody or complement (86). In the mouse system, primary (87,88) and secondary (89) cytotoxic cell responses have been demonstrated following MLC. Primary (90,91) and secondary (92) MLC-induced CTL have also been found in human cell cultures. The effector cell in the mouse system is an Ly 2+, 3+

T cell (63) and a OKT5+/8+ (or Leu 2+) T cell (66) in humans. A recent report (93) which phenotypically characterizes the human MLC-generated cytotoxic cell, describes the presence of the activation antigen $4F_2$ on the CTL and a lack of receptors for IgG on this cell.

Eijsvoogel et al. (90,94) showed in their studies that although disparity at HLA-D is necessary for the generation of CTL, the antigens recognized by the CTL are those coded by the HLA-A,B and C regions. It has also been shown that differences at D were not absolutely necessary for CTL induction (this is analogous to experiments in mice showing that CTL can develop with only a K region difference)(95). Although HLA-A,B and C determinants appear to serve as important targets for CTL, recent work by Feighery and Stastny (96,97) demonstrates that CTL could develop in MLCs between HLA-D mismatched, HLA-A,B matched individuals that were directed against the specific HLA-D product. Strong cellular cytotoxicity to I-region determinants has also been described in the mouse CTL system (98,99). It is presently not clear whether the products recognized by CTL are the same as those seen by serological techniques. The use of in vitro generated cytotoxic T-lymphocytes as typing reagents is currently under way in several labs and a recent European CML workshop (100) was held to compare cellular typings of the HLA locus. Since the techniques are presently available to clone and propagate lymphocytes in vitro for long periods of time, the feasibility of CTL as typing reagents is increasing.

Generation Of "Primed" Or Memory Lymphocytes During The MLC

Following the initial burst of cell division in the primary MLC, the clones of sensitized lymphocytes begin to slow down metabolically and revert back to cells with the morphology of small, resting

lymphocytes. This is a heterogeneous population which contains specifically primed cytotoxic cells (discussed above) and other memory cells which also show secondary response kinetics. The primed cells, as mentioned before, display an accelerated proliferative response toward the specific primary stimulator cell and this forms the basis of the PLT assay. The nature of the stimulating antigens involved in the PLT response has been previously discussed. The PLT cell appears to be the helper or responder (63,66) cell which proliferates and aids in the development of cytotoxic cells during the MLC.

Regulation of the MLC by Suppressor Cells

Rich and Rich (101) first described in the mouse the generation of cells from alloantigen challenged mice which inhibited syngeneic MLC responses in vitro. It was subsequently shown by several investigators that the mouse MLC was itself capable of generating suppressor cells which could inhibit other MLC and CTL responses (102,103,104). The mechanism of the suppression appeared not to be due to killing of the responders (101) although this claim has been disputed (105). The cell responsible for suppression is a T cell with the Ly2+,3+ phenotype (62,63). These negatively regulatory cells have also been described in human MLC reactions (106,107,108,109). One group claims that the suppressor T cell in their system is nondiscriminatory in its suppression (106), while others find preferential suppression occurring with cells autologous to the suppressor at HLA-D (107,108). Although the mechanism of suppression is unclear, there are reports claiming that suppression is mediated by soluble factors (110,111). Recently, the human MLC-induced suppressor cell has been demonstrated to be found largely in the fraction of T lymphocytes identified by the Leu 3 antibody (69).

This is an interesting finding since the Leu 3 subset is thought to correspond to the "helper" T cell population in man while suppression in other systems have been associated with the Leu 2 population.

Engleman et al.(112,113) have described the presence of suppressor cells in the blood of several individuals who did not respond in MLC to specific stimulator cells. This radiation-sensitive cell appears to only suppress responses of autologous cells or cells which share HLA-D with itself (thus, it is genetically restricted at HLA-D). Additionally, the suppressor cell shows specificity in that only the responses to specific alloantigens are suppressed. This genetically restricted, specific suppressor cell has also been reported by other labs (114,115). These latter descriptions of suppressors are of interest since they may give some insight into the controls that the HLA region has on normal immune function and in the role of various cell subsets in autoimmune and other disease processes.

The Autologous MLC

In 1975 Opelz et al.(116) made the surprising discovery that when human T lymphocytes were incubated with autologous B lymphocyte-enriched cell populations, a significant response was measured at 6 days. These results were corroborated in humans (117,118) and mice (119) and the phenomenon became known as the autologous MLC. Although the responder cell has consistently been found to be a T cell, studies by Sakane and Green (120) have shown that the T cells responsive to autologous cells are largely separable from T cells responsive to allogeneic cells. The nature of the stimulator cell, as in the allogeneic MLC, is currently a matter of controversy. While many labs claim B cells (116,121) and K (null) cells (117) to be the major stimulating cell populations, most

have not used purified preparations. Recently, MacDermott and Stacey (122), using highly purified cell populations, showed that B cells were poor stimulators whereas macrophages possessed the most potent stimulating capacity in the autologous MLC. Other studies have shown that the autologous MLC, and not the allogeneic MLC can be inhibited by physiologic doses of hydrocortisone (118,123). Finally, the findings of decreased autologous MLC activity in lupus patients (124,125), and the generation of suppressor cells during the autologous MLC (126) suggest that this phenomenon may be part of the normal autoregulating mechanisms present in the immune response.

Clinical Significance of the MLC

Early studies on the MLC suggested that the test could serve as a method of evaluating the degree of histocompatibility between two individuals. Since this could be a valuable tool in transplantation medicine (predominantly transplants of kidneys and bone marrow) numerous studies have been done on the relevance between the MLC reaction and the survival of the graft.

What has emerged is a confusing picture on the usefulness of the MLC in regards to transplantation. The most beneficial role of the MLC appears to be almost exclusively limited to living related donor transplants, especially among HLA-identical siblings. These serologically-identical individuals rarely react to each other in MLC (95) and also have the greatest chance for graft survival (127). When evaluating 1-haplotype mismatch MLCs, however, often the reactions seen are as high as 2-haplotype MLCs. Dupont et al.(95) and others (128) have shown tremendous overlap when analyzing the means of a considerable number of

1-haplotype versus 2-haplotype MLCs. This brings up the basic problem that one encounters in MLCs; that is, correlating the degree of proliferation in the MLC (which is often high in the 1-haplotype MLCs) with the suitability of the prospective donor (or the histocompatibility difference between the donor and recipient). Some labs have claimed correlation between the MLC and graft survival by using cutoff stimulation indices (129) or relative responses (130). However, several reports exist claiming no correlation using the same means of data analysis (131,132).

In conclusion, the emergence of the MLC as a clear-cut analytical tool in transplantation has not yet occurred and its role in the future in this regard certainly has to be evaluated further.

II. Cytokines and the MLC

The role of soluble cytokines in the primary proliferative phase of the MLC is unclear, although the MLC has been shown to induce production of many of these molecules. An attempt will be made to briefly summarize characteristics of several lymphokines and monokines which may have a significant influence on the MLC, although many of the studies were performed with mitogen-stimulated cultures or in other systems. A distinction between the human and mouse systems will be made only when necessary.

Mitogenic Factors (MF)

Many of the presently described lymphokines and monokines can be termed "mitogenic factors" since they stimulate cell division of lymphocytes. This section will describe, however, substances which induce

mitogenesis of mature, unstimulated lymphocytes, as opposed to antigen-stimulated cultures.

Kasakura and Lowenstein (133) reported in 1965 the appearance of soluble mitogenic factor in the supernatants of human MLCs which induced resting lymphocytes to proliferate. The peak of MF production was found 3 to 5 days after initiation of the MLC and irradiation appeared to have little effect on the release of MF (134). T cells proliferate in response to MF stimulation (135,136). Uotila et al. (137) suggested that the cytotoxic T cell is the target while Kasakura (138) has shown the development of nonspecific cytotoxic T cells after exposure to MF. Others have claimed that B cells are also stimulated to divide by MF (139,140). MF is produced by T cells (141) requiring macrophages (142) to help initiate MF production. The regulation of MF levels has been suggested to be under the influence of suppressor cells (143,144) which when removed, allow for enhanced MF activity in the supernatants. The need for antigen in order for MF to work is presently unclear. Using MF derived from lymphocytes stimulated with tetanus toxoid, Geha and Merler (145) found that MF function was only expressed in the presence of antigen. However, others have found no genetic restriction of MLC-derived MF, suggesting no role for solubilized transplantation antigens (146).

The molecular weight (mw) of MF has been reported from being a 80,000 mw protein tetramer (140) to a 20,000 to 28,000 mw protein (139). However, extensive biochemical analysis of MF is lacking. It is currently unknown whether the activities attributed to MF and several described human cytokines (such as IL2, see below) are performed by the same or separate molecules. Another consideration is whether it is

wise to compare MF studies since a careful evaluation of the biochemical and functional characteristics of MFs from different sources (MLCs, mitogens, antigens) is not available.

Most of the studies on MF described above were done with supernatants from human cell cultures. Few factors derived from mouse cells have been found to stimulate mature, resting cells. One well described mitogenic factor in the mouse system is allogeneic effect factor (AEF), which was first reported in 1974 by Armerding and Katz (147). The method of production of AEF is uncommon in the sense that it is the product of a secondary in vitro MLC following a primary in vivo alloantigen priming by thymocytes. The original activity designated to AEF was its ability to replace T helper cells in augmenting the in vitro antibody responses by B cells (147). Subsequently, molecular characterization of AEF revealed the presence of Ia determinants on the molecule (148,149). The most recent and interesting biologic effects of AEF are the stimulatory signals that AEF gives to mature resting T cells. It has been shown that AEF has the capacity to induce the development of cytotoxic T cells which preferentially lyse autologous cells (150). Additionally, AEF causes the proliferation of mature T cells which when tested after priming, react in secondary kinetics to autologous cells (151). Thus, this product of alloantigen-activated T cells appears to preferentially induce autoreactive effector cells. The physiological significance of this process is presently unknown.

T Cell Growth Factor (TCGF) or Interleukin 2 (IL2)

In 1976, the discovery was made that supernatants or conditioned media from PHA-stimulated mononuclear cell cultures could support the

growth in vitro of human T cells activated by lectin (152). This growth factor appeared in mononuclear cell supernatants of T cells stimulated with either mitogens or alloantigens (153,154). It was later shown that T cells produced this factor (155); in particular, the source of TCGF was helper T cells, possibly in a macrophage independent process (156). A quantitative microassay was developed for the measurement of T cell growth factor (153) using long term cytotoxic T cell lines dependent on TCGF for their growth (157). In mice, it appears that normal, resting T cells do not respond to TCGF (155,158,159,160), and that the acquisition of responsiveness to TCGF is a direct result of the T lymphocyte activation by mitogen or alloantigens. The need for a cell to be activated in order to respond to TCGF has also been demonstrated by the inability of resting cells to absorb out any TCGF activity in the mouse (155) and human (161) systems, whereas activated cells absorb TCGF very efficiently. Cytotoxic T cells are the primary cells which respond to TCGF (155,159), and the expression of receptors for TCGF on these cells occurs as early as 4 hours following cell-lectin interaction (162). It has recently been shown that cytotoxic cells may also be capable of producing TCGF when restimulated with H-2 K/D region antigens (163).

Several mouse and human tumor lines have been found that produce large amounts of TCGF following stimulation with mitogen or phorbol myristate acetate (164,165,166). Biochemical analysis reveals that murine TCGF has a molecular weight of 30,000 (167) while rat and human TCGF have a molecular weight of 15,000 (168). In addition to its unique property of propagating cultured T cell lines, Watson et al. (167,168) found that the TCGF column fractions had other biological

properties. These properties are the ability to augment thymocyte responses to submitogenic doses of mitogen (costimulator function), helper T-cell replacing factor (TRF) activity in athymic spleen cell cultures and the induction of cytotoxic lymphocytes in both thymocyte and nude mouse spleen cell cultures. Another property of TCGF is that steroids (169) and cyclosporin A (170) inhibit the production but not the effect of this lymphokine. These drugs may serve as probes to investigate molecular mechanisms involved in TCGF's effect. These and other studies on TCGF have suggested that this molecule may be the only mitogenic signal generated during cell-lectin interaction, and that it is intricately involved with a macrophage product known as LAF (lymphocyte-activating factor) or IL-1 (discussed below). Smith et al. (171) have demonstrated that the addition of LAF containing supernatants increases the levels of TCGF that is produced and thus increases T cell proliferation. From this they have proposed that LAF serves as a signal from the macrophage (which has bound the lectin) to help initiate and augment TCGF production, which eventually controls the level of the clonal T cell response.

Lymphocyte Activating Factor (LAF) or Interleukin I

Macrophages secrete a large number of products which modulate the immune response. These substances range from small molecular weight molecules such as prostaglandins (172) and free radicals (173), to larger molecules (usually proteins) which are collectively known as monokines. One monokine described by Gery et al. (174) possessed potent mitogenic properties for thymocytes, but would minimally stimulate peripheral T cells, and was named lymphocyte activating

factor (LAF). The source of LAF was shown to be adherent cells rather than lymphocytes (174,175). The stimulant used by Gery to produce LAF was bacterial lipopolysaccharide (LPS), an agent known to activate macrophages. Other stimulants (including the MLC) found to induce LAF from human leukocytes or murine peritoneal exudate cells, have also been known to activate macrophages either directly or indirectly through lymphocytes (for review, 176). Several macrophage tumor lines produce LAF (177) when stimulated by LPS, and Mizel et al.(178) find the molecular weight of LAF from the P388 macrophage line around 15,000 mw. Earlier molecular weight descriptions of LAF have been similar (174,179), with some investigators also finding high molecular weight forms of LAF (179). No size differences have been found between human and murine LAF.

Some biological properties of LAF include the previously mentioned thymocyte mitogenic ability, the capacity to replace macrophages in the induction of cytotoxic T cells (180), and the enhancement of the B cell antibody response (181). A most interesting finding is the increased production of TCGF by addition of LAF. As discussed previously, these studies imply that LAF or Interleukin I is a signal generated during lectin-macrophage interactions which aids in potentiating T cell proliferation through the production of TCGF. The role that these two "interleukins" have in the immune response, although still ill-defined, is clearly an important one. The relationship that these products have to other cytokines is currently an area of intense interest.

SPECIFIC AIMS

According to the clonal selection theory (182), lymphocytes proliferate and expand into specific sensitized clones when exposed to antigens. In the case of the MLC, the belief is that the clonal proliferation of specific T cells exposed to allodeterminants on the stimulator cells determines the magnitude of the response, implying that the higher the number of antigens mismatched between the responder and stimulator, the higher the MLC response.

However, comparison of many 1-haplotype and 2-haplotype MLCs shows that although the overall mean of the responses in 1-haplotype MLCs is lower, the overlap between the two groups is substantial. Since these results do not sustain the concept that greater HLA-D disparity equates with higher MLC reactivity, a series of studies were initiated to identify which factors other than HLA-D might affect the magnitude of the proliferative response in MLCs.

These studies addressed the following areas.

1. Examination of the overall or "general" stimulating ability and responsiveness of MNL.
    a. Peripheral blood MNL from HLA-identical siblings were used to stimulate different responder cells to determine whether there was any difference in their stimulatory ability. These potential differences would not be related to HLA-D.

b. Individual stimulator cell populations were compared with pools of stimulators for their capacity to stimulate in MLC. These studies were done to determine the stimulation of one stimulator cell population relative to a pool of stimulators with multiple HLA-D disparities present.

c. Individuals who were used in several MLCs were analyzed for their "relative" stimulating ability to determine whether some persons were consistently better stimulators than others in MLCs.

d. Mitogen assays were simultaneously performed with MLCs to determine whether an association of responsiveness could be established in different assay systems.

2. A major effort was dedicated to better define MLC-derived soluble mitogenic factors (MLC-MF) and to explore the possibility that they may be an important source of nonspecific proliferation in the MLC.

a. Studies were done to optimize conditions for the procurement and production of sufficient quantities of MLC-MF with high levels of activity.

b. The levels of MLC-MF in MLC supernatants were measured and compared to the response of the original MLC (where the supernatant was taken) in an attempt to correlate MLC-MF production with the MLC response.

c. The effect of supernatants from MLCs containing MLC-MF activity was tested in autologous and allogeneic MLCs.

d. Studies were performed to determine the nature of the cell which was providing a "helper" effect to cells responding to MLC-MF.

e. Studies were done to find out whether the proliferative effect of MLC-MF was mediated through further production of mitogenic factors or whether the effect on MNL was direct on responder cells.

f. Experiments were also designed to explore whether soluble alloantigens could be involved in the effect of MLC-MF.

g. The cells responding to MLC-MF were also analyzed using monoclonal antibodies to T cell subsets.

h. In column chromatography and absorption experiments the relationship between MLC-MF and IL2 was investigated.

These investigations led to the identification of a number of variables that can significantly influence the outcome of a MLC. In addition, they definitively established stimulatory properties of cells independent of HLA-D and a clarification of the way in which MLC-induced mitogenic factors exert their nonspecific effects on lymphocytes.

MATERIALS AND METHODS

Individuals. All the studies were performed using cells obtained from normal individuals and in some cases with end stage renal disease (ESRD) patients. The behavior in MLC of the cells from ESRD patients as a group was similar to the cells from normal individuals.

Cell preparations. All of the cell preparations and assays were performed sterilely.

Human mononuclear leucocytes (MNL). Human peripheral blood was drawn in heparin (Upjohn, Kalamazoo, MI, 10 I.U./ml). The buffy coats were removed, layered over Ficoll-Hypaque and MNL isolated by centrifugation (183). The MNL were washed three times (1600 rpm, 10 minutes, room temperature) with phosphate buffered saline (PBS) and resuspended in RPMI-1640 (GIBCO, Grand Island, NY) supplemented with 50 ug/ml gentamycin (Schering, Kenilworth, NJ) and 20% pooled human AB serum (Bio-bee, Boston, MA). This medium (complete) was used for most assays unless otherwise noted.

Non-adherent (NA) human MNL. Human peripheral blood MNL (isolated as described above) were run through a series of steps in order to obtain the population of cells referred to as nonadherent cells. The first step consisted of incubation of 10 X 10^6 MNL in 100 X 15 mm plastic petri dishes (Falcon, No. 1029) in 5 ml of complete medium for 1 hour at 37°C. Duplicates were run according to the number of cells

needed. Following the incubation, the plates were examined microscopically to ensure that cells were attaching, and the non-adherent cells were rinsed off, using 3 washes per plate. These non-adherent cells were then pooled and isolated by centrifugation (1600 rpm, 10 minutes, room temperature). The supernatant was decanted and the cells were resuspended in .5 ml of complete medium at 37°C. The cells were then added to nylon wool columns which had been equilibrated to a temperature of 37°C. The columns were removed, and medium was run through to elute the non-adherent cells. Subsequently, the cells were centrifuged, resuspended and counted.

The proportion of lymphocytes and monocytes before and after separation was determined by electronic sizing in a Coulter channelizer and Coulter Counter Model 2 ZI. Approximately 10^5 cells were placed in 10 ml of Isoton Plus and 0.77 ml of Lyse S Plus (both from Coulter Diagnostic, Hialeah, FL) and aspirated into the Coulter Counter. The size distribution was recorded into a Hewlitt-Packard 9845T computer and the proportion of the large and small cells was calculated according to the areas of the corresponding peaks. The large cells (monocytes) are practically eliminated by sequential attachment to plastic surfaces and nylon wool. Monocyte concentration was estimated by myeloperoxidase staining (184).

MLC-primed cells (PLT). These cells were obtained by incubating 10 X 10^6 responder MNL from one individual plus 10 X 10^6 irradiated MNL from another individual in a volume of 12 ml of complete medium in 75 cm^2 flasks (Corning, New York, NY) for 10 days at 37°C. Following this period, the cells were collected, washed twice, and viable cells counted.

Murine thymocytes. Thymuses were removed from 4 to 6 week old C57BL/6 mice, cut and pressed through a nylon mesh screen, and run through a series of varying gauge (19,21,23 and 25) needles to obtain a single cell suspension. The cell suspension was then overlayed on Ficoll Hypaque, and centrifuged at 1200-1300 rpm for 15 minutes at room temperature. The pelleted cells (or high density thymocytes) were then removed, washed 3 times with PBS and resuspended in medium used for the costimulator assay (see below), and counted. Phytohemagglutinin (PHA)-stimulated MNL were prepared by incubating MNL at a concentration of 1 X 10^6 cells/ml in complete medium with .1% PHA-P (Difco, Detroit, MI) for 3 days at 37°C, 5% CO_2, in 75 cm^2 tissue culture flasks (Corning). These cells were collected, washed 3 times with PBS and counted.

Cryopreservation and thawing of human MNL. Human MNL were isolated from peripheral blood as described above with the exception that the MNL were resuspended after the final wash in RPMI-1640 supplemented with 20% newborn calf serum (NCS) (GIBCO) and antibiotics. After counting the cells were adjusted to a concentration of 6.0 X 10^6 cells/ml with the same medium. An equal volume of RPMI-1640 containing 20% DMSO and 20% NCS was then added to the cell mixture at the approximate rate of 1 ml/minute. This step was done on ice and the final cell concentration was now 3.0 X 10^6 cells/ml. The cell suspension was then added in 1.0 ml aliquots to freezing ampules (Nunc, Intermed, Roskilde, Denmark) kept on ice. The freezing of the cells was done according to a method described by Dr. Rene Duquesnoy in a personal communication. Briefly, the ampules were transferred to a styrofoam box which had multiple holes in it. The covered box was then put in a -70°C freezer

for 24 hours (the holes served the purpose of allowing the temperature within the box to gradually decrease). After 24 hours, the ampules were transfered to liquid nitrogen, where they were kept until used in MLC.

Frozen cells were thawed in the following manner. The ampules were removed from the liquid nitrogen, placed in a 40°C water bath for 60-90 seconds until thawed, and then immediately placed on ice. The 1.0 ml of cells was then transferred to a larger tube and 9.0 ml of RPMI-1640 with 20% NCS was added at a rate of 1 ml per minute. The cells were then spun down, resuspended, and viable cells enumerated.

In Vitro Assay Systems

Mixed lymphocyte cultures (MLCs). (Figure 1) MLCs were performed in round-bottom 96 well plates (Dynatech, Alexandria, VA), each well containing 0.2 ml of RPMI-1640 supplemented with 20% human AB serum and gentamycin (complete medium). The wells also contained 30 X 10^3 responder MNL and, unless noted, 120 X 10^3 stimulator MNL irradiated with 2500 rads from a ^{137}Cs source (Gammator Model M). The plates were then incubated at 37°C, 5% CO_2, for a total of 6 days. On day 5, the cultures were pulsed with 1.0 uCi of ^3H-thymidine (Schwarz/Mann, specific activity of 6 Ci/mM) and harvested 18 hours later onto filter papers with a 24-line harvester (Otto Hiller, Madison, WI). The filters were air dried, placed into vials with an appropriate volume of scintillation fluid and counted in a LKB Model 81000 liquid scintillation counter.

MLC-MF assay. (Figure 2) Supernatants from MLCs were routinely tested for their mitogenic activity on resting human MNL in 96 well

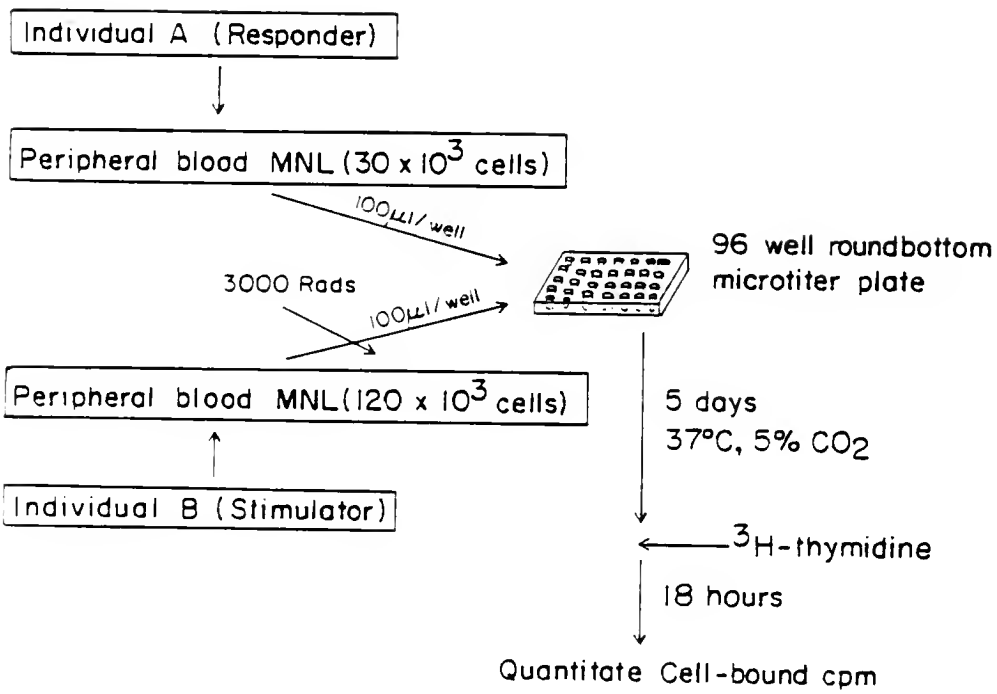


Figure 1. Human mixed lymphocyte culture (MLC).

round-bottom plates. 30 X 10^3 responder MNL in 0.1 ml of complete medium were incubated with 0.1 ml of MLC supernatants in each well. The cultures were incubated for 6 days at 37°C in 5% CO_2. On day 5, cultures were pulsed with 1.0 uCi/well of ^{3}H-thymidine and harvested 18 hours later with a 24 line harvester. Filters were collected and counted as described in the MLC assay. When testing for helper cell activity, 120 X 10^3 autologous, irradiated (3000 rads) unseparated or NA MNL were added with the responder cells at the beginning of the incubation period. In some experiments, hydrocortisone sodium succinate (HC) (Upjohn) was added initially to some wells in 10 ul volumes at different concentrations and allowed to remain for the duration of the experiment.

On several occasions PLT cells were also used as responder cells to MLC supernatants. The assay consisted of 30 X 10^3 PLT cells in 100 ul of complete medium in the presence of 100 ul of supernatant. Pulsing was done after 48 hours at 37°C and harvesting 72 hours after initiation of the cultures.

Costimulator assay. MLC supernatants and column fractions were tested for their ability to enhance mitogen (at suboptimal dose) induced responses in mouse thymocytes (185). 5 X 10^5 C57BL/6 thymocytes in RPMI-1640 supplemented with 1.0% fresh autologous mouse serum, mercaptoethanol (5 X 10^{-5} M), 10 mM HEPES buffer, 100 I.U./ml penicillin, 100 ug/ml streptomycin and .25 ug/ml Fungizone (GIBCO) were added in a total volume of 100 ul to 100 ul of MLC supernatant or column fraction, with or without 0.3 ug/ml of Conconavalin A (ConA) (Miles-Yeda, Rehovot, Israel). After 48 hours, cultures were pulsed with 0.5 uCi/well of ^{3}H-thymidine and harvested 24 hours later.

T cell growth factor (TCGF) assay. Levels of TCGF in supernatant or fraction samples were determined by the stimulatory activity of these samples on a long term, cultured human T cell line (CTCl). Thirty thousand (30 X 10^3) CTCl cells in 100 ul were incubated at 37°C with 100 ul of the sample being tested. The cultures were pulsed with 1.0 uCi of ^{3}H-thymidine at 24 hours and harvested 48 hours following the initiation of the incubation.

The CTCl cell line was derived from a 10 day old MLC. Following the initial MLC, the cells have been propagated in vitro by the addition of unrelated, irradiated MNL to the culture flasks in the presence of mitogenic doses (.1%) of PHA-P. Six days prior to the TCGF assays, the cells are maintained only by the addition of supernatants from MNL stimulated by PHA. At this time, the cells are usually exclusively responsive to TCGF and not to PHA. Assays were not considered in which the CTCl cells responded to PHA.

Mitogen assays. MNL from patients and controls were also used in proliferation assays using three well known mitogens, PHA, ConA, and pokeweed mitogen (PWM) (Sigma, St. Louis, MO). Briefly, the responding MNL were used at 30 X 10^3 cells per well (in round-bottom microtiter plates) in a volume of 100 ul. The mitogens were also added in 100 ul with the optimal concentrations being .1% for PHA, 0.6 ug/ml for ConA and 12 ug/ml for PWM. After 48 hours incubation at 37°C, the cultures were pulsed with 1.0 uCi/well of ^{3}H-thymidine and harvested 18 hours later as described in the assays above.

Production of MLC-MF (Figure 2). Mixed leucocyte cultures were set up in 17 X 100 mm tubes (Falcon, Oxnard, CA) using 3.0 X 10^6

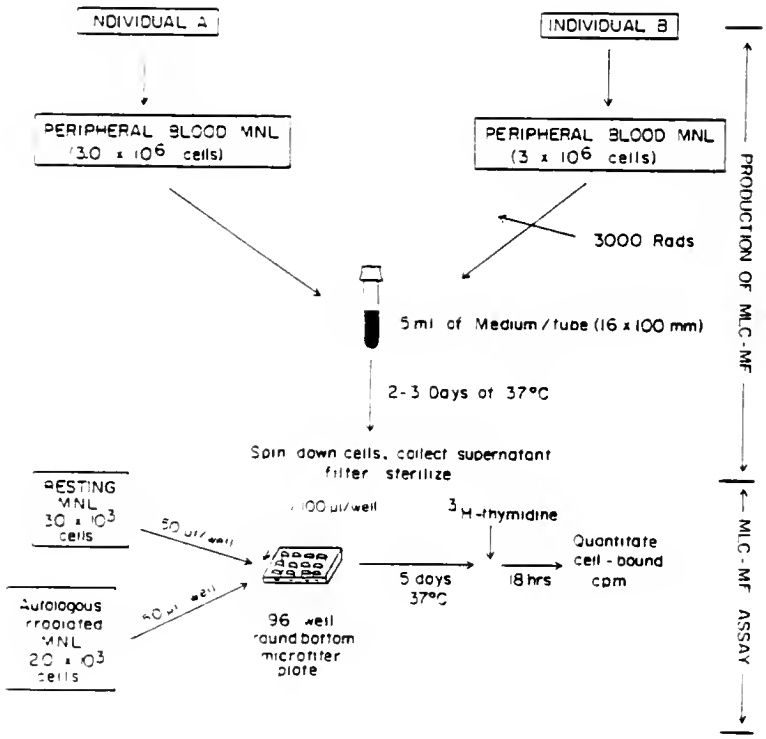


Figure 2. MLC-MF: Production and assay.

responder MNL and 3.0 X 10^6 irradiated stimulator MNL (2500 rads) in a total volume of 5 ml of complete medium (RPMI-1640 with 20% human AB serum). After 48-72 hours, the tubes were mixed, centrifuged (2000 rpm, 10°C, 10 minutes) and the cell-free supernatants removed. All supernatants were pooled, filtered through a .45 um filter (Sybron/Nalge, Rochester, NY), aliquoted and kept frozen at -70°C until used. In some experiments, hydrocortisone, prepared in PBS, was added initially to cultures at several concentrations and allowed to remain in the supernatant when collected. On several occasions, PLT cells were specifically restimulated on day 10 with the original stimulator cell and supernatants were collected 48-72 hours later.

Absorption of MLC supernatants. Human and murine tumor lines, resting MNL and PHA-stimulated MNL were used for absorption at concentrations from 20 X 10^6 per ml of MLC supernatant. The human tumor line was a T cell lymphoma that is terminal deoxyribotransferase and leu 1 (Becton Dickinson, Oxnard, CA) positive (kindly provided by Dr. Raul Braylan). The mouse tumor line used was M3, a methylcholanthrene-induced fibrosarcoma derived from C57BL/6 mice (kindly provided by Dr. Paul Klein). PHA-stimulated and resting MNL were prepared as previously described. Absorptions were performed at 4°C for 30 minutes (to prevent the release of factors from the absorbing cells), mixing every 10 minutes. After the incubation, the tubes were spun down in a refrigerated centrifuge, and the MLC supernatants were removed, sterilized through a .45 um filter and kept frozen at -70°C until used.

Treatment of MNL with OKT antibodies. Unseparated and NA MNL were treated with the monoclonal antibodies OKT4 and OKT8 (Ortho, Raritan,

NJ), and rabbit complement (Pel-Freeze). Thirty microliters of each antibody was added to 3.0 X 10^6 MNL which were in a volume of 170 ul of RPMI alone. The reactions were done sterilely in 12 X 75 mm tubes at 4°C for thirty minutes, agitating every 10 minutes. Following this incubation, 200 ul of complement was added to each tube and the reaction allowed to proceed for 60 minutes at 37°C. After this step, the tubes were spun down (1400 rpm, 10°C, 10 minutes) and washed twice with PBS. Finally, the cells were resuspended in complete medium and the viable cells counted.

Column chromatography. MLC-MF fractionation was accomplished by Sephadex G100 chromatography (100 X 2.6 cm column; pH 7.4 PBS, constant flow elution of 15 ml/hour) of the MLC supernatants after 50-70 fold concentrations (Diaflo ultrafilter PM10). Standards for molecular weight estimation included bovine serum albumin, lysozyme and ovalbumin (Sigma).

HLA typing. Typing for the HLA-A and B locus antigens was done with the microcytotoxicity technique (186) with antisera obtained from the National Institutes of Health and from local sources by screening with a panel of 30 cells of known phenotypes. DR typing was performed with the double immunofluorescence technique (187). DR antisera was obtained from Dr. Terasaki's laboratory at the University of California at Los Angeles.

RESULTS

I. Studies of Stimulation in the Human MLC

Establishment of the Optimal Conditions for the MLC

Several studies were done to determine optimal and practical parameters of the MLC. An analysis of the responder cell in the MLC indicated that 30 X 10^3 MNL consistently provided responses which were as good as higher numbers of cells (data not shown). In studies of the stimulator cell concentrations, numerous cell titrations were used with 30 X 10^3 responders of which several are shown in Figure 3. It was determined that 120 X 10^3 MNL induced an excellent response in most MLCs. The use of this responder/stimulator cell ratio of 30 X 10^3/ 120 X 10^3 also had the advantage of being a small number of cells, which would allow for a greater number of variables to be analyzed with the limited cell numbers often obtained from some patients and normal individuals. Optimal MLC responses were also found when pooled human AB serum was used at 10-20% concentration.

Experiments were also performed to determine whether preincubation of responders and stimulators significantly affected their respective activities. As shown in Figure 4, the ability of cells to respond slightly increased when they were preincubated for 4 days. Cells left in culture for 2 to 4 days and then irradiated, showed an enhanced ability to stimulate in comparison with fresh cells. On the other hand,

Figure 3. Varying numbers of stimulator cells in MLCs. Each line represents a different MLC combination in which the stimulator cells were used at four cell concentrations (30, 60, 120 and 240 X 10^3 cells). Each point represents the mean of triplicate values.

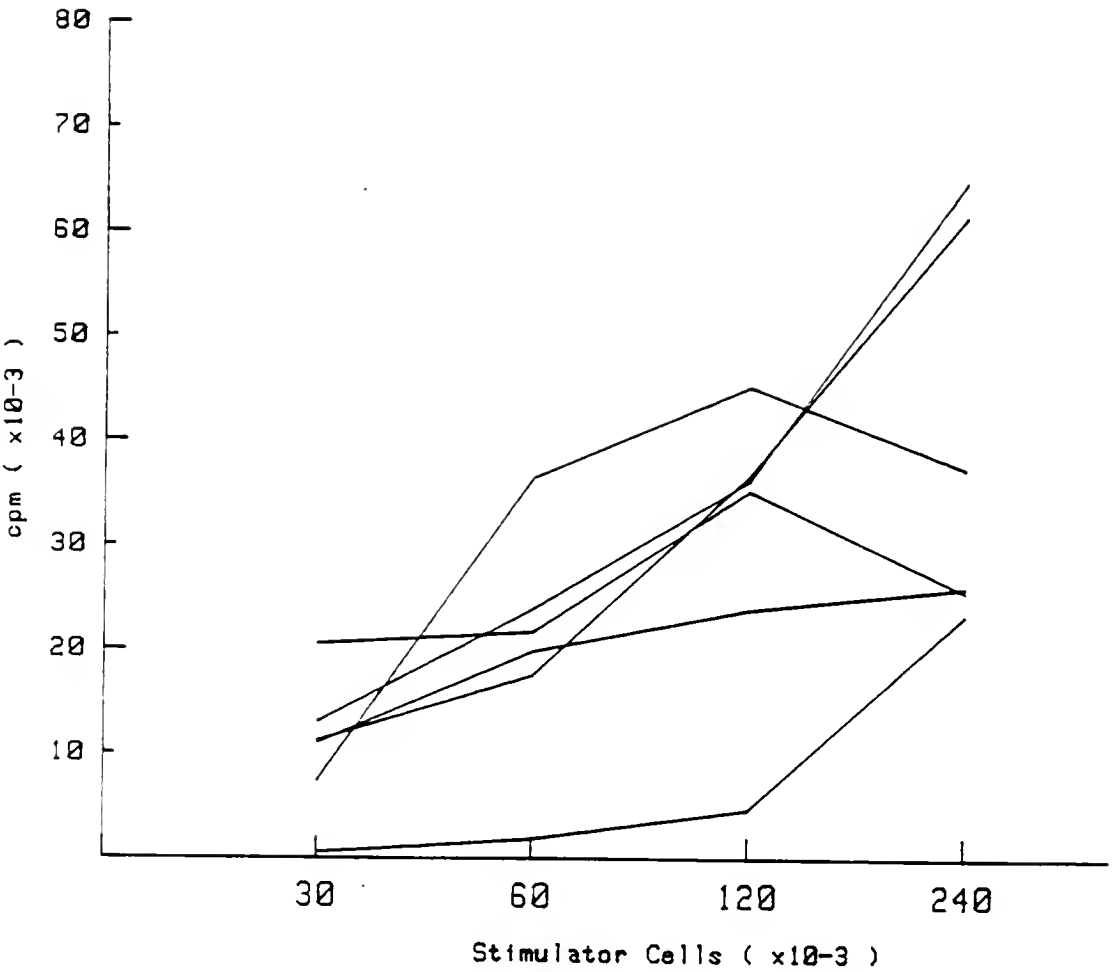
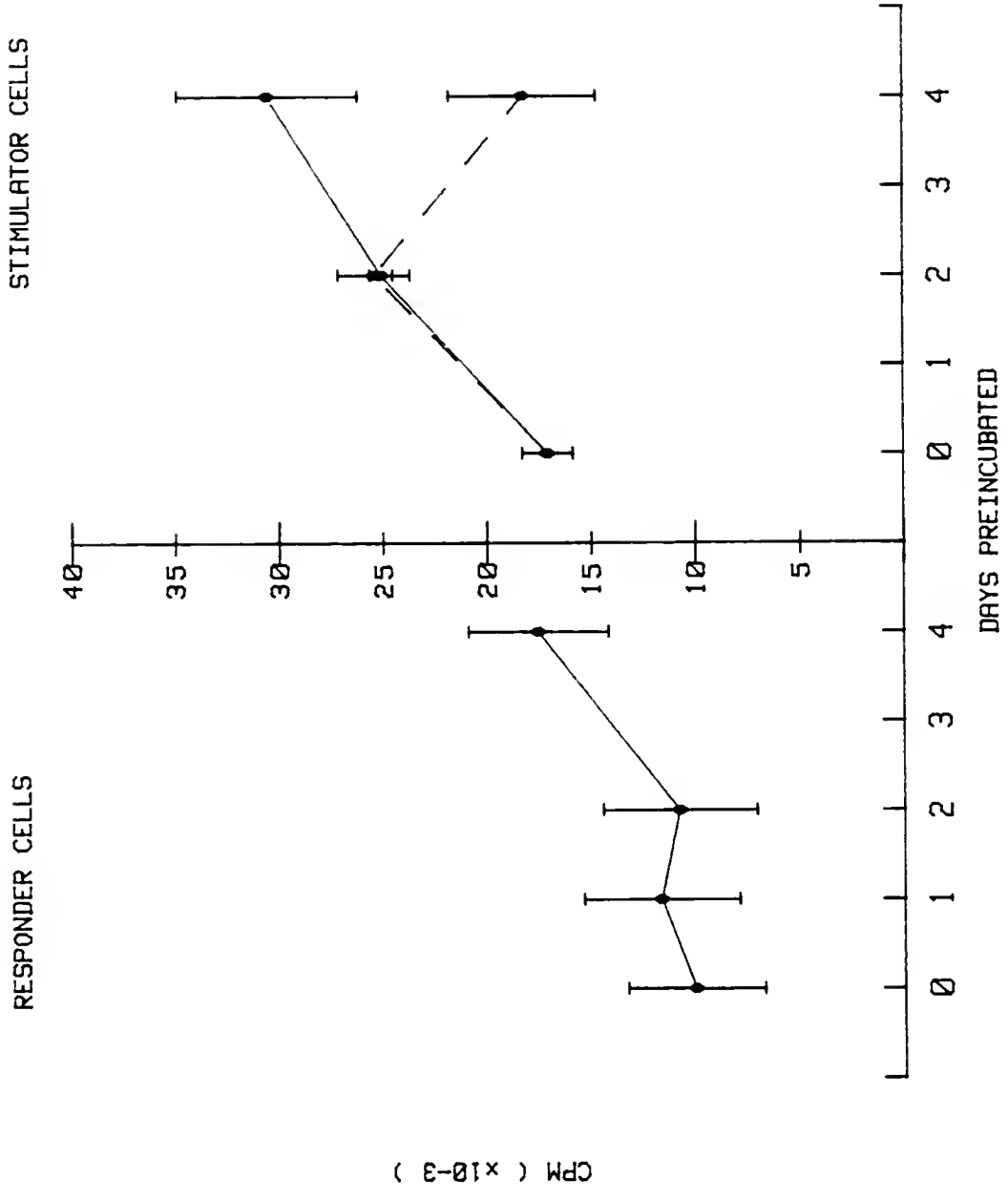

80
70
60
50
40
30
20
10
cpm (x10-3)
30
60
120
240
Stimulator Cells (x10-3)

Figure 4. Effect of preincubation on responsiveness and stimulation in the MLC. The left hand side of Figure 4 compares the ability of cells to respond in MLC when left in culture for several days to the response of fresh cells. This was accomplished by sequential bleeding of the same individual for 4 days. The person's cells were also used to study the effects of incubation on the ability of the cells to stimulate (right side). The solid line are cells left in culture for the described number of days and irradiated on the day the responders were added. The dotted line are cells irradiated on the day they were put in culture. Each point represents the mean of triplicate values $\pm$ the S.D.

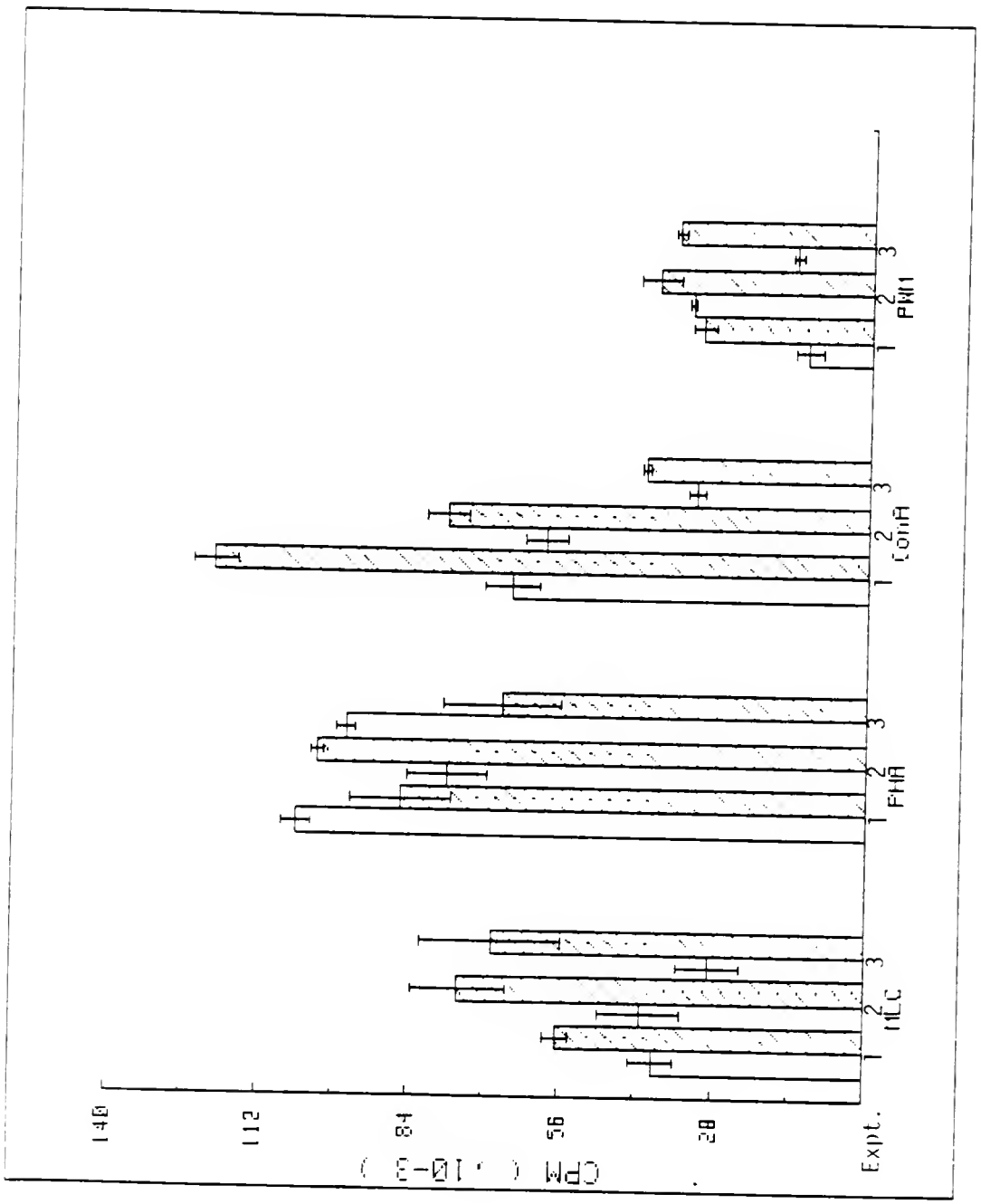

STIMULATOR CELLS
RESPONDER CELLS
40
35
30
25
20
15
10
5
0
1
2
3
4
DAYS PREINCUBATED
CPM (x10-3)

irradiated cells left in culture had less ability to stimulate than non-irradiated cells left in culture for the same time period.

Studies of Individuals (Patients and Controls) with Plant Mitogens and Mixed Lymphocyte Cultures

Experiments were initiated to attempt to evaluate the general immune responsiveness of patients and normal individuals. Peripheral blood MNL were isolated and used simultaneously in both MLCs (against a panel of ten stimulators) and in assays with the mitogens, PHA, ConA and PWM. In all cases, patients were analyzed with normal individuals as controls and often the same patient-control pairs would be studied on several occasions. Figure 5 shows a representative study of the responses of a patient and control in three separate experiments using the 10 stimulator cell-panel MLCs and the mitogen assay systems. It can be noted that a significant degree of variability exists from one experiment to the next when using the mitogens, ConA and PHA, while the smaller responses seen with PWM were less variable. The mean of the 5 highest MLC responses was used as the method to represent the general alloresponsive state of the individual. In the study shown and in others performed, the MLC was consistently less variable than the mitogen responses. For example, by calculating the mean of the 5 highest MLC values in 3 experiments, Figure 5 shows that the patient (open bars) was always a lower responder than the control. PWM did induce a lower response by the patient in all 3 experiments, however this consistency was not found in other studies. ALso apparent is the lack of correlation between the MLC and the mitogen response. In many situations, the highest responses to mitogens by an individual could not be associated with a similar high MLC response by his cells.

Figure 5. Mixed lymphocyte cultures and mitogen assays of two individuals in three separate experiments. This figure describes the responses of the MNL of a patient and control in three separate experiments in 4 different assays run simultaneously (MLC, PHA, ConA, and PWM). The open bars represent the response of the patient and the striped bars those of the control. The numbers beneath each pair of bars designates which experiment these two values represent. Thus, the values for Experiment 1 in all 4 assays can be compared since they were done at the same time. The numbers for the mitogen assays represent the mean of triplicate values $\pm$ the S.D. The values for the MLC are actually the means of the 5 highest MLC responses to a panel of ten individual stimulator cells (i.e., the responder MNL were tested to 10 different stimulator cells).


CPM (.10-3)
140
112
84
56
28
Expt.
MLC
PHA
ConA
PWM
1
2
3

MLC Stimulation by a Panel of Unrelated MNL

ESRD patients and controls were evaluated for their ability to respond in MLCs to panels of stimulator cells (frozen). Figure 6 shows that responder 1 (open bars) was clearly a lower responder than responder 2 (striped bars) to every stimulator cell tested. Analysis of these and other experiments allowed the observation that populations of MNL from many individuals induced either high or low stimulation with most of the other responder cells used in that MLC. This is exemplified in Figure 6 in which cells which induced high stimulation in responder 1 were also a good stimulator for the other responder. Statistical analysis of the responses of these two individuals (Figure 7) in 4 independent MLCs to the same panel of cells indicated that in fact there is a correlation of both responses to a given stimulator cell. Since both responders were reactive to each other in MLC, occasional similarities at the D locus with a given cell of the panel would be expected to produce less stimulation in one responder but not in the other. The results shown in Figure 7 suggest that this is not the case and also that the degree of stimulation was unrelated to the concentration of monocytes or B cells. These results, which were representative of a trend seen in three other similar experiments, suggested that cells display a high or low general stimulatory activity to unrelated cells.

MLC Stimulation by HLA-Identical Cells to a Panel of Responder Cells

The following experiments confirmed the previous suggestion and established that the different capacity to stimulate in MLC was unrelated to different degrees of HLA-D disparity.

Figure 6. Mean of 4 separate MLC responses to a panel of cryopreserved stimulator cells and a pool of those cells by an ESRD patient (open bars) and a control (striped bars). Each letter designates a stimulator cell (frozen) used in each experiment, and each bar represents the mean $\pm$ S.D. of the 4 MLC responses to each stimulator cell.

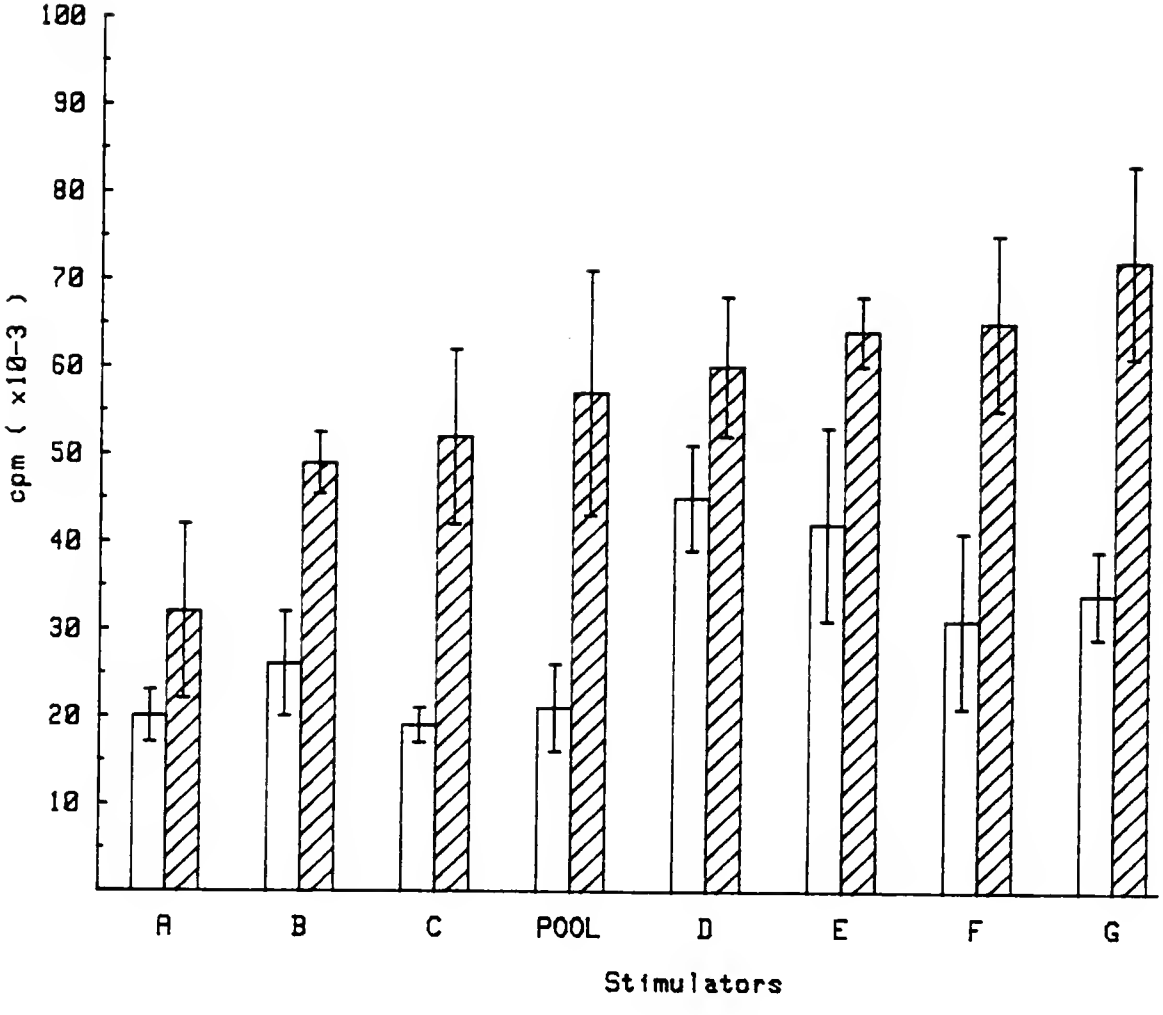
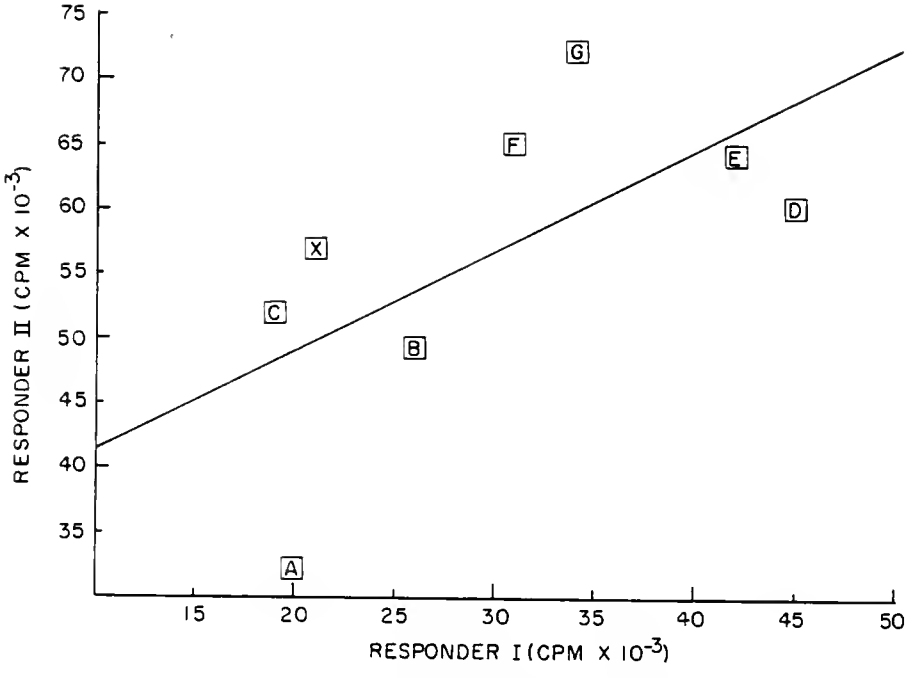

100
90
80
70
60
50
40
30
20
10
cpm (x10-3)
A
B
C
POOL
D
E
F
G
Stimulators

Figure 7. Correlation between the responses of an ESRD patient (Responder 1) and a normal control (Responder 2) induced by a panel of cryopreserved normal stimulator cells. The correlation observed ($r = .62$) was at the limit of statistical significance ($p = .051$). Each letter refers to each individual stimulator cell, X being a pool of all of them. The values that they represent in the graph are means of 4 independent experiments performed with the same participants at different times. The coefficient of variation was less than 20% except for X versus responder 1 (25%), E versus responder 2 (26%) and F versus responder 2 (28%). Autologous controls were under 2500 cpm. The response of responder 1 stimulated by responder 2 was 20,000 to 38,000 cpm in the different MLCs and responder 2 versus 1 was 31,000 to 49,000 cpm.



	A	B	C	D	E	F	G
Monocytes %	Nd.	28	22	26	26	36	20
B Cells %	Nd.	8	9	4	5	4	7

The first experiment shown (Figure 8) compared the ability of cells from 2 normal HLA-identical siblings to behave as stimulators to a panel of ten unrelated individuals as responders. Neither of the siblings responded to each other in MLC, indicating identity at HLA-D. It can be seen that for every responder tested, one of the siblings was a consistent low stimulator whereas the other was a reasonably good stimulator. In the same experiment, cells from both siblings were added together as stimulators with results consistent with summation of individual effects rather than suppression by the low stimulator. Both siblings were used in another MLC with two unrelated responders and similar results were obtained, the same sibling stimulating low responses on both occasions.

The next experiment (Figure 9) shows the response of four unrelated individuals to stimulator cells from identical twins. Again, one of the siblings stimulated all responders significantly better than the other. In this experiment, monocytes and B cells were enumerated. The proportion of monocytes, as estimated by myeloperoxidase staining and Coulter sizing, was similar in both twins. The proportion of B cells was slightly higher in the sibling that produced lower stimulation. HLA and red cell antigens were identical for both twins (see legend for Figure 9). Figure 9 also shows that the response of each sibling to different stimulators did not correlate with their capacity to stimulate in MLC, a fact that we observed repeatedly in other MLCs.

Finally, Figure 10 shows the same phenomenon with two normal HLA-identical siblings stimulating other members of the family. One of the siblings was capable of inducing higher responses from all the family members than the other sibling. Identity at HLA-D between the siblings

Figure 8. Stimulation by two HLA identical individuals to a panel of unrelated responders. Each letter designates a different responder cell. Stimulator and responder cells were all obtained the same day of the experiment. Each pair of bars corresponds to the stimulation produced by the 1st (striped) and 2nd (open) sibling to a given responder cell. Lines in each bar indicate standard deviation of triplicate cultures. Both siblings were HLA-A3,29, HLA-B22,44, HLA-DR7. Autologous control of 1st sibling was 743 cpm $\pm$ 121 and the response to her sibling was 381 $\pm$ 13. Autologous control of the 2nd sibling was 1061 $\pm$ 218 and the response to her sibling was 613 $\pm$ 135.

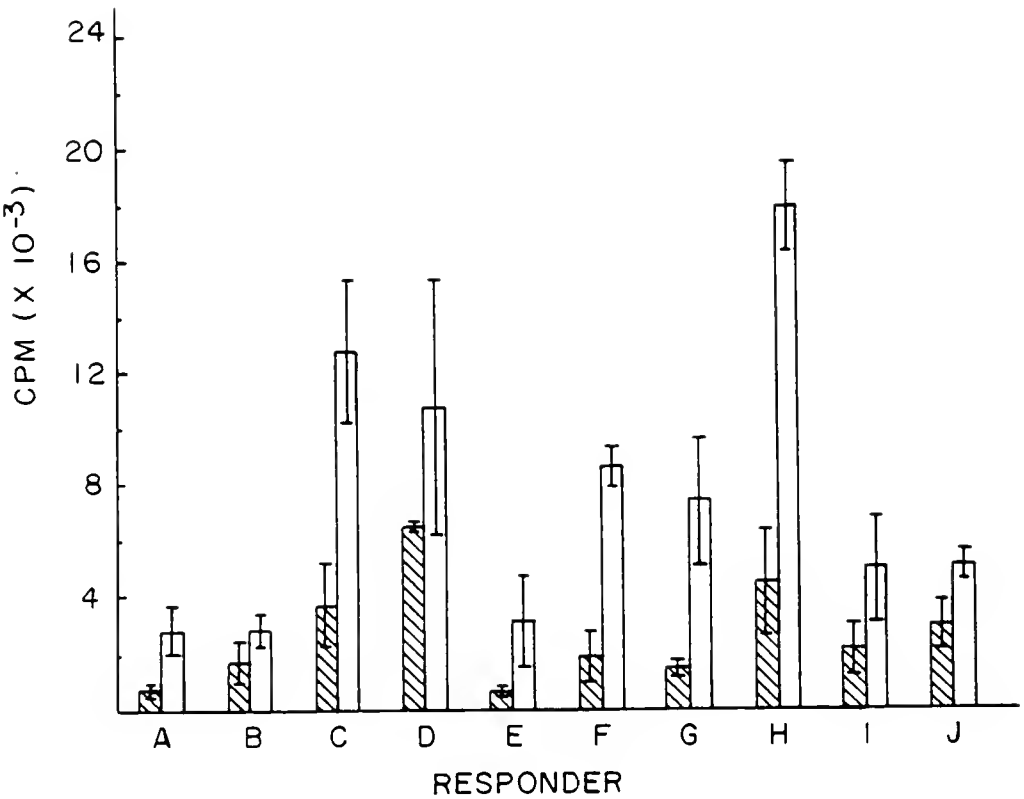
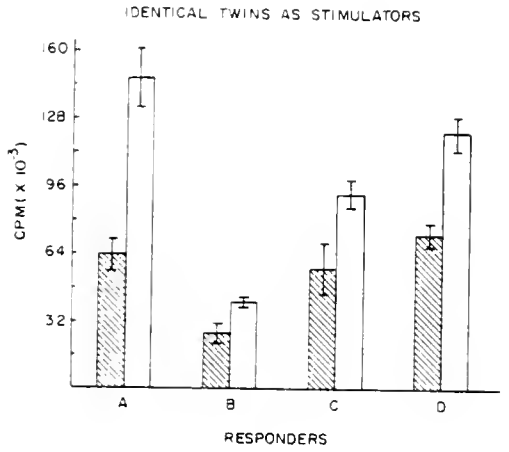

24
20
16
12
8
4
CPM (X 10-3)
A
B
C
D
E
F
G
H
I
J
RESPONDER

Figure 9. Stimulation and response of two normal identical twins versus unrelated cells. Top: Each pair of bars indicates the stimulation produced by the cells from the 1st (striped) and the 2nd (open) twin to the corresponding responder. Bottom: Each pair of bars indicates the response of each twin to the corresponding stimulator. All cells were obtained the same day of the experiment. Lines in each bar indicate standard deviation of triplicate cultures. Both twins, in addition to obvious physical and behavioral similarities, shared HLA-A11,26, B14,22 DR2,3 antigens as well as red cell antigens (ABO, Rh complex, MNS, P, K, Fy and Jk). Autologous control of the 1st twin was 2400 cpm ± 1122 and the response to her sibling was 3015 ± 991. Autologous control of the 2nd twin was 1521 ± 166 and the response to her sibling was 1874 ± 738. Myeloperoxidase positive MNL in the 1st and 2nd twins were 6 and 9% respectively, monocytes quantitated by Coulter sizing were 9 and 11% and B cells were 9 and 5% respectively.


IDENTICAL TWINS AS STIMULATORS
160
128
96
64
32
CPM (x 10⁻³)
A
B
C
D
RESPONDERS

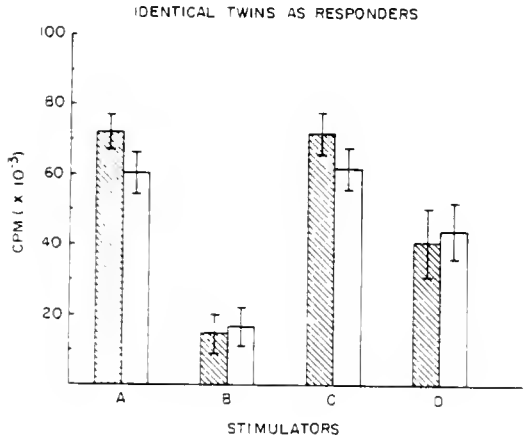
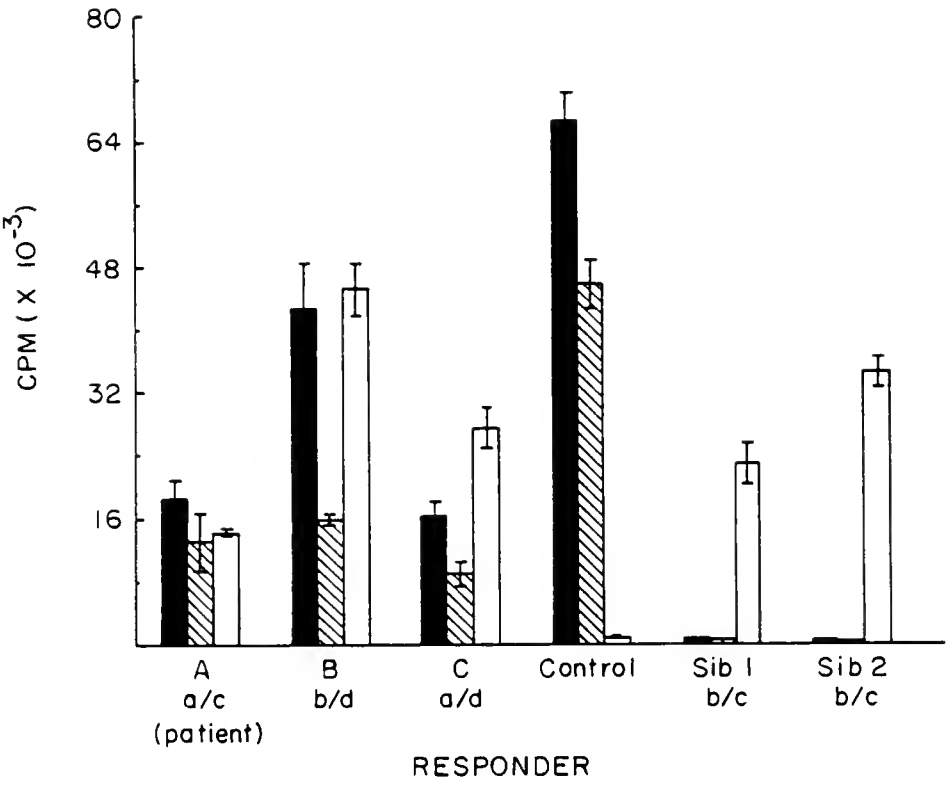

IDENTICAL TWINS AS RESPONDERS
100
80
60
40
20
CPM (x 10⁻³)
A
B
C
D
STIMULATORS

Figure 10. Stimulation induced by two HLA-identical siblings on other members of the family. A is an ESRD patient, B is a patient's sister, C, a sister, and the control is an unrelated individual. Sib 1, a sister, and Sib 2, a brother, are the two HLA-identical siblings also used as stimulators. The solid bars are stimulation by sibling 1, striped bars are stimulation by sibling 2 and open bars are stimulation by the unrelated control. Lines in the bars are standard deviation of triplicate cultures. The haplotypes are: a:A1,B8, b:Aw31,Bw35, c:A2,B7, d:Aw24,B7. DR typings were not done.


80
64
48
32
16
CPM (X 10^{-3})
A
a/c
(patient)
B
b/d
C
a/d
Control
Sib 1
b/c
Sib 2
b/c
RESPONDER

used as stimulators was established due to a lack of reaction in MLC between them. Thus, the different stimulatory activity of HLA-identical individuals is seen not only with unrelated responders, but also with one and two haplotype mismatch intrafamilial combinations.

Individuals with "High" and "Low" Stimulatory Ability

The sibling which induced low stimulation in Figure 8 is a laboratory technologist that participated in a number of MLCs as a normal control for ESRD patients and relatives. The relative response induced by this person's cells on the responders of each MLC (it is actually "relative stimulation") is shown in Table 1 (Individual 2). It can be seen that in six of the seven MLCs the "relative stimulation" was less than 1.00, suggesting that this individual is a low stimulator. In contrast, the same analysis performed in another individual that was also studied in several MLCs as a control showed that the relative stimulation was higher than 1.00 in six out of nine MLCs (Table 1, Individual I). A third individual analyzed in the same manner produced results intermediate between the other two.

Responses to Stimulator Pools

The existence of high stimulators was also shown in experiments comparing the response to single stimulators with the response to pools of two stimulators. The response of cells (30×10^3) from one individual was tested with a panel of unrelated stimulator cells (60 and 120 $\times 10^3$). The same responder was also tested with several pools of two stimulator cells (60×10^3 each) in a way that if the stimulators are A through K, the pools were formed by A-B, A-C, A-D, etc. Table 2 shows

TABLE 1

INDIVIDUALS INDUCING HIGH OR LOW STIMULATION IN MLC

Individual 1		Individual 2	
Experiment #[a]	Relative Stimulation	Experiment #	Relative Stimulation
1	2.09 ± .48[b]	10	.83 ± .21
2	2.29 ± 1.20	11	.45 ± .01
3	.84 ± .21	12	.52 ± .27
4	1.14 ± .09	13	1.67 ± .80
5	2.95 ± 1.30	14	.32 ± .21
6	.53 ± .10	15	.77 ± .04
7	1.30 ± .38	16	.82 ± .25
8	.81 ± .08		
9	2.26 ± .24		
Average[c]	1.57 ± .83		.76 ± .44

(a) Experiments with Individuals 1 and 2 were not performed simultaneously.

(b) This value was obtained in the following way. The MLC was done with a patient (A), relative (B), control (C) and individual 1 as a second control (D). Results were as follows:

Response of A to DX: 56,604 cpm (a)
Response of A to either B,C or pool, whichever was higher: 29,756 cpm (b) Ratio (a/b) = 1.90

Response of B to Dx: 67,044 cpm
Highest response of B to A, C or pool: 38,452 cpm Ratio = 1.74

Response of C to Dx: 54,658 cpm
Highest response of C to A, B or pool: 20,585 cpm Ratio = 2.65

Avg ± S.D. = 2.09 ± .48

(c) The relative stimulation produced by Individual 1 and Individual 2 was significantly different ($p = 0.037$) in a two sided t test for identity of population means.

TABLE 2

RESPONSE TO INDIVIDUAL STIMULATORS AND POOLS OF 2 STIMULATORS IN MLC

Single Stimulators						Pools		
				cpm				
Individual	DR[a]	B Cells (%)	Monocytes (%)	60 X 10^3 Cells	120 X 10^3 Cells	Stimulators (60 X 10^3 each)	cpm	Pool/Highest[b] Ratio
A	5,7	n.d.[c]	n.d.	50546	55974			
B[d]	1,-	8	28	22549	31444	A + B	64029	.88
C	4,-	n.d.	n.d.	36410	51481	A + C	55653	.77
D	n.d.	6	24	29935	59088	A + D	67582	.93
E	4,5	4	36	35563	51228	A + E	65726	.91
F	n.d.	n.d.	n.d.	34030	44841	A + F	62076	.86
G	1,w6	6	12	35563	46364	A + G	73136	1.01
H	2,-	7	20	21866	43243	A + H	68541	.95
I	w6,7	10	24	39254	72382	A + I	71838	.99
J	n.d.	n.d.	n.d.	33206	48410	A + J	81546	1.13
K	5,-	9	33	26403	39561	A + K	68715	.95

[a] Responder cells were DR 2,3.

[b] The highest value used to calculate this ratio was individual I with 120 X 10^3 cells.

[c] Not done.

[d] Except for the responder and individual A, all other stimulator cells were cryopreserved.

one representative experiment out of three performed. It can be seen that while most of the pools stimulated more than the individual stimulators alone (presumably as a consequence of additional HLA-D disparities) there was one individual (I in Table 2) which produced a stimulation higher than most of the pools studied. These results show that one good stimulator, with a maximum of two HLA-D disparities with the responder, can induce a response higher than that produced by three or four disparities. Individual high stimulators were also more effective than pools of 10 stimulators. Table 3 shows twenty MLCs in which the best of 10 individual stimulators was compared to a pool of all them. In each MLC there was at least one stimulator that stimulated equal or better than the pool.

II. Investigations on a Mitogenic Factor Induced During the MLC

The above results indicated that the ability to stimulate in the MLC was possibly influenced by non-genetic factors present during the course of an MLC. One of these non-genetic variables that was investigated was a blastogenic or mitogenic factor generated during the MLC (MLC-MF).

Effect of MLC Supernatants (MLC-MF) on Normal Resting MNL

The initial studies on MLC-MF utilized supernatants collected on day 5 from MLC microtiter plates. These supernatants were added to MLC (e.g. ABx) combinations to see if there was an increase in the response, especially in the case of low responders. The results shown in Figure 11 are typical of what occurs when MLC supernatants are added to allogeneic MLC combinations. In most cases there is no effect when

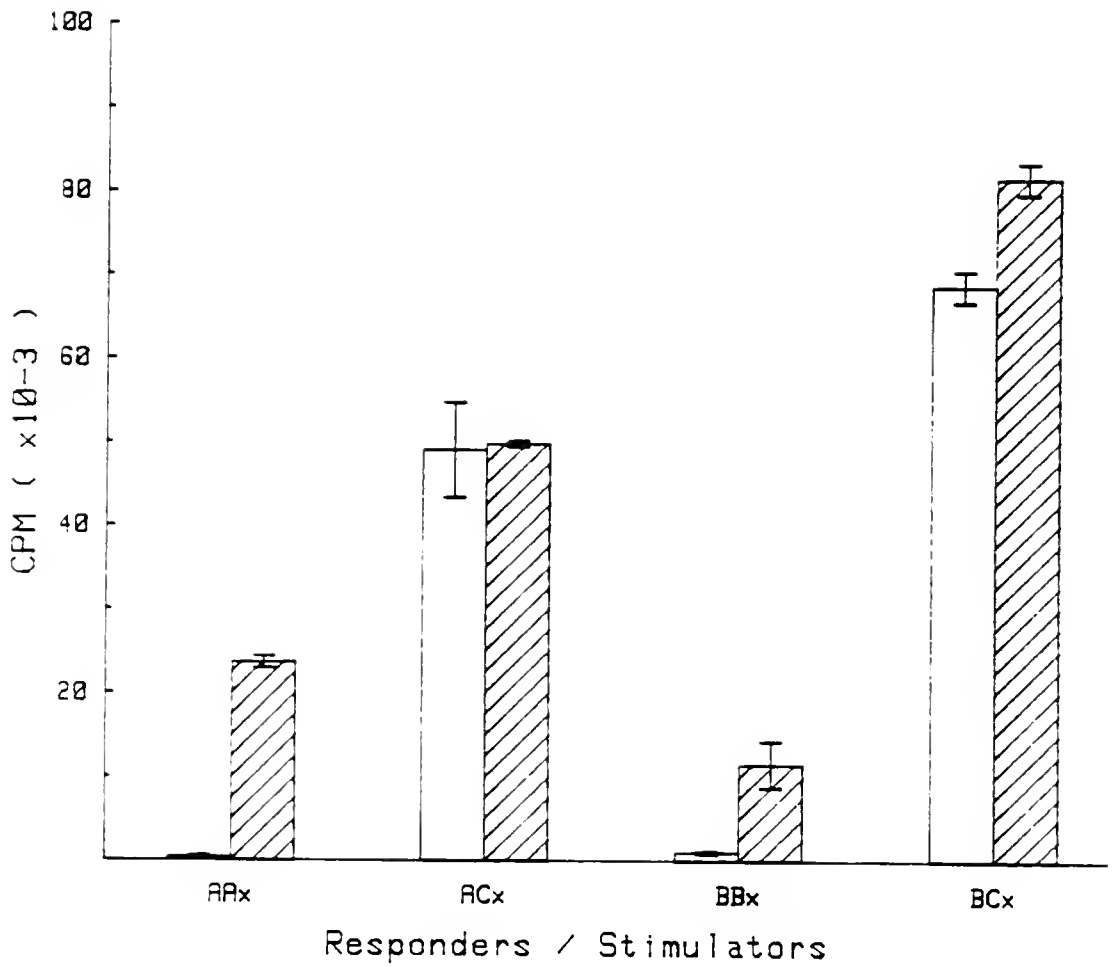
TABLE 3

RESPONSE TO A PANEL OF TEN STIMULATORS AND TO A POOL OF TEN STIMULATORS

Experiment	Responder	Pooled Stimulators (cpm)	Highest Response to One Stimulator (cpm)	Pooled/Highest Ratio
1	A[a]	37807	51548	.73
2	A	42976	46238	.93
3	B	54620	72215	.76
4	B	46915	58660	.80
5	B	77662	83961	.92
6	B	48870	84811	.58
7	C	40019	45234	.88
8	C	56157	59020	.95
9	D	43265	43496	.99
10	E	12979	12325	1.05
11	E	21910	32194	.68
12	F	24027	55254	.43
13	F	23456	43089	.54
14	F	31292	51524	.61
15	F	22326	38035	.59
16	G	92948	99434	.93
17	H	80176	75416	1.06
18	H	49738	56262	.88
19	I	74932	73097	1.03
20	I	26663	38166	.70

(a) The same letter refers to the same individual studied in different opportunities. Stimulator cells were cryopreserved.

Figure 11. Effect of MLC supernatants on autologous and allogeneic MLCs. MLC supernatants added at 50% concentration to MLC combinations (ACx and BCx) and to the autologous controls (AAx and BBx). The open bars represent the values when no MLC supernatant was added and the striped bars the values seen when MLC supernatant was added. Each bar represents the mean of triplicate values $\pm$ the S.D.


100
80
60
40
20
CPM (x10-3)
AAx
ACx
BBx
BCx
Responders / Stimulators

measured at 6 days and rarely is the response augmented. However, there was a greatly increased response at 6 days in the autologous or control combination (AAx) when the MLC supernatant was added (Figure 11). For example, the response of AAx is above 20,000 cpm in the presence of MLC-MF and approximately 500 cpm without.

These MLC supernatants were also tested for their capacity to stimulate MNL from a patient who had Severe Combined Immunodeficiency (SCID) and MNL from a patient with hypogammaglobinemia. The results in Table 4 indicate that MNL from the SCID patient failed to respond MLC-MF and also in the MLC. In contrast, there was no deficiency in the ability of the SCID patient's cells to stimulate in MLC. The MNL from the patient with hypogammaglobinemia responded well in MLC and to MLC-MF (as did the controls) and also served as good stimulators in MLC.

The effects of the MLC supernatants on normal resting cells was the impetus for the work described below.

Establishment of Conditions for the Production of MLC-MF

The MLC-MF preparations made in plates used in the initial experiments had the disadvantages of having low activity, of small volumes, of being tedious to procure, and of taking 5 days to obtain. Studies were thus planned on improving the preparatory conditions, volume and activity of MLC-MF. In a time course experiment (described below, Figure 16) it was found that the peak of MLC-MF appeared from 2 to 3 days following initiation of the MLC. Using this information, "macro" MLCs were set up in 16 X 100 mm tubes using variable numbers of responders to stimulators in different volumes and each combination was run as either a 0, 1 or 2 way MLC (0 way = both cell populations

TABLE 4

RESPONSE OF A PATIENT WITH SEVERE COMBINED IMMUNODEFICIENCY (SCID) TO MLC-MF

A = SCID Patient
B = Patient with Hypogammaglobinemia
C = Normal Control
D = Normal Control

Combinations	cpm ± S.D.[a]
A(30) + Ax	420 ± 147
" + Ax + MLC-MF[b]	940 ± 655
" + Cx	397 ± 98
" + Cx + MLC-MF	401 ± 15
" + Dx	251 ± 83
" + Dx + MLC-MF	242 ± 120
B(30) + Bx	804 ± 59
" + Bx + MLC-MF	3,335 ± 38
" + Cx	6,281 ± 3,340
" + Dx	18,294 ± 173
C(30) + Cx	985 ± 323
" + Cx + MLC-MF	7,999 ± 624
" + Ax	6,789 ± 1,591
" + Bx	1,710 ± 1,029
" + Dx	19,309 ± 7,911
D(30) + Dx	600 ± 17
" + Dx + MLC-MF	3,368 ± 1,259
" + Ax	3,388 ± 1,114
" + Bx	4,333 ± 2,740
" + Cx	12,961 ± 2,785

[a] cpm = counts per minute ± standard deviation

[b] MLC-MF was added at 50% concentration

irradiated; 1 way = one cell population irradiated; 2 way = neither population irradiated). Table 5 shows that a significant increase in the MLC-MF activity of MLC supernatants was obtained in most of the tube combinations when compared to the plate supernatants. Of special significance were the 5 ml volume tubes with 3.0 X 10^6 cells of each cell population (culture no. 8, Table 5). The high activity obtained in the one-way MLC combination showed that a large volume of MLC-MF could be prepared by setting up multiple tubes in the same combinations with an accessible number of cells. Also of interest is the high MLC-MF activity seen in 0-way combinations which indicates that irradiated cells are very capable of producing MLC-MF. Two-way MLC combinations actually contained the least activity. This was presumably due to consumption of MLC-MF during the proliferation occurring in some cells early in the MLC.

The supernatants were also tested for their mitogenic activity on cells which had been stimulated with PHA for 6 days. These activated cells did not further respond to PHA but would to TCGF-containing supernatants. As shown (Table 5), many of the supernatants induced proliferation of these cells. An analysis of the association between the actions of these supernatants on resting cells and on the PHA-stimulated cells indicated a significant correlation between the two activities (Figure 12).

Comparison of MLC Response with MLC-MF Activity of Supernatant

A study was performed to determine whether an association existed between the degree of proliferation measured in the MLC and the amount of MLC-MF that could be measured in the supernatant. Table 6 shows the

TABLE 5

ESTABLISHMENT OF CONDITIONS FOR PRODUCTION OF MLC-MF

MLC CULTURE CONDITIONS[a]				SUPERNATANT ACTIVITY[b]	
Culture No.	No.of Cells A(X 10^6)	No.of Cells B(X 10^6)	Volume	0,1,or 2 way MLC	Fresh Cells (MLC-MF)	PHA Stimulated Cells
1	.15	.60	1 ml	ABx	36,757 ± 4,403	2,809 ± 1,574
	"	"	"	AB	19,298 ± 4,260	1,564 ± 174
	"	"	"	AxBx	13,397 ± 8,974	1,326 ± 126
2	.30	.60	1 ml	ABx	39,531 ± 4,791	3,441 ± 476
	"	"	"	AB	17,590 ± 7,302	2,120 ± 186
	"	"	"	AxBx	23,332 ± 11,528	3,332 ± 453
3	.60	.60	1 ml	ABx	27,299 ± 3,532	3,326 ± 427
	"	"	"	AB	14,895 ± 3,690	1,755 ± 365
	"	"	"	AxBx	33,371 ± 7,156	5,088 ± 203
4	1.2	.60	1 ml	ABx	N.D.[c]	N.D.
	"	"	"	AB	21,511 ± 7,373	2,970 ± 536
	"	"	"	AxBx	41,269 ± 2,547	4,197 ± 421
5	2.4	.60	1 ml	ABx	N.D.	N.D.
	"	"	"	AB	26,805 ± 14,000	3,077 ± 513
	"	"	"	AxBx	35,076 ± 4,120	3,615 ± 871
6	1.2	1.2	1 ml	ABx	N.D.	N.D.
	"	"	"	AB	26,366 ± 2,641	3,594 ± 584
	"	"	"	AxBx	43,798 ± 2,301	5,931 ± 1,091
7	2.4	2.4	1 ml	ABx	27,008 ± 6,482	3,395 ± 1,120
	"	"	"	AB	9,620 ± 965	1,803 ± 650
	"	"	"	AxBx	39,558 ± 5,742	5,032 ± 608

Table 5 Continued

8	3.0	3.0	5 ml	ABx	36,207 ± 5,008	3,523 ± 632
	"	"	"	AB	19,369 ± 860	4,163 ± 810
	"	"	"	AxBx	22,905 ± 4,044	3,232 ± 753
9	6.0	6.0	5 ml	ABx	34,474 ± 8,350	6,186 ± 1,020
	"	"	"	AB	22,763 ± 1,200	4,347 ± 749
	"	"	"	AxBx	40,215 ± 5,005	3,970 ± 194
10	.03	.12	.2 ml	ABx	13,442 ± 1,810	1,396 ± 47
	"	"	"	AB	7,728 ± 1,377	N.D.
	"	"	"	AxBx	651 ± 460	N.D.
11	--	1.2	1 ml	B	1,226 ± 298	N.D.

(a) Cultures 1-9 and 11 were done in 16 X 100 mm tubes, while culture 10 was done in 96 well microtiter plates. The incubation time for all cultures was 2 days at 37°C, 5% CO_2 in complete medium (20% AB serum). Following this period, the tubes and plates were spun down to pellet the cells and the supernatants were removed, filter sterilized and kept at -70°C until assayed.

(b) MLC-MF activity was detected by testing the collected supernatants at 50% concentration on 30 X 10^3 fresh MNL plus 120 X 10^3 irradiated, autologous MNL and measuring the response at 6 days (MLC-MF assay). The supernatants were also tested on cells which had been stimulated with PHA for 6 days (and no longer responded to PHA). The incubation time in this experiment was 3 days. The background cpm for the fresh cells was 1,806 ± 640. The background cpm for the PHA-stimulated cells was 1,028 ± 294 and in the presence of .1% PHA it was 1,729 ± 218.

(c) Not done.

Figure 12. Statistical analysis of correlation between the activity of MLC supernatants on resting cells and on PHA-activated cells (values obtained from Table 5). The data show that there was correlation since the linear correlation coefficient was significantly different from 0 with P = .00000021. Values on both the x and y axis are measuring cpm.

Correl. coeff. (r)	.31
Sample size (n)	28.00
't' statistic	6.97
Degr. freedom	26.00

	one-sided test	two-sided test
p value	.00000011	.00000021
confidence level (%)	100	100

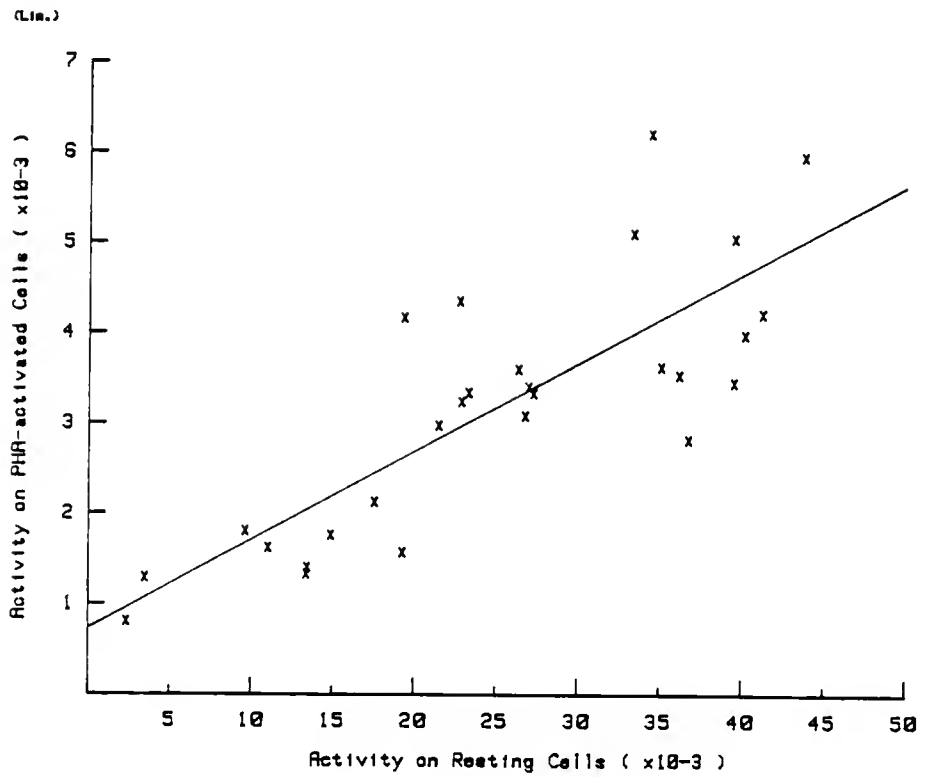


TABLE 6

COMPARISON OF MLC RESPONSE WITH MLC-MF ACTIVITY OF SUPERNATANT[a]

Combination #	MLC Response	MLC-MF Activity of MLC Supernatants	
		Responder 1	Responder 2
1	4,663 ± 3,875	1,902 ± 1,086	899 ± 360
2	81,019 ± 2,540	14,904 ± 3,582	11,942 ± 3,004
3	48,372 ± 9,954	6,884 ± 1,150	10,012 ± 2,254
4	50,024 ± 8,121	10,392 ± 4,541	5,256 ± 234
5	5,170 ± 1,950	1,009 ± 142	1,550 ± 797
6	50,020 ± 6,589	4,222 ± 2,715	4,228 ± 503
7	27,598 ± 3,396	7,428 ± 2,804	1,644 ± 940
8	32,457 ± 4,058	1,689 ± 1,036	1,387 ± 71
9	4,437 ± 2,630	1,197 ± 688	777 ± 168
10	93,922 ± 4,941	12,870 ± 1,135	11,276 ± 1,171
11	47,104 ± 11,515	13,529 ± 739	12,652 ± 3,011
12	41,728 ± 11,254	11,840 ± 902	7,386 ± 3,052
13	13,580 ± 2,245	1,808 ± 727	420 ± 189
14	64,676 ± 5,057	8,382 ± 3,672	3,827 ± 1,551
15	52,701 ± 1,769	4,215 ± 1,449	4,360 ± 3,076
16	32,130 ± 11,235	1,709 ± 508	1,804 ± 770
17	644 ± 196	519 ± 250	1,031 ± 663
18	28,056 ± 3,423	8,370 ± 1,713	6,036 ± 926
19	39,073 ± 1,965	8,763 ± 1,834	6,986 ± 1,727
20	23,956 ± 2,252	5,386 ± 1,085	5,327 ± 2,928
21	1,408 ± 692	895 ± 261	1,210 ± 450
22	19,762 ± 5,036	2,487 ± 218	1,310 ± 582
23	24,279 ± 5,065	3,269 ± 1,902	1,649 ± 663
24	8,385 ± 3,412	3,837 ± 1,737	2,252 ± 1,998
25	1,286 ± 1,382	3,597 ± 403	1,183 ± 695
26	74,019 ± 12,687	6,763 ± 2,269	5,062 ± 1,184
27	88,356 ± 9,192	9,663 ± 1,416	4,707 ± 1,489
28	55,596 ± 6,278	7,114 ± 2,005	6,479 ± 1,208
29	2,741 ± 834	4,146 ± 2,538	1,387 ± 927
30	37,500 ± 6,515	5,167 ± 1,051	6,344 ± 3,678
31	55,120 ± 9,005	3,496 ± 1,616	3,929 ± 1,962
32	33,910 ± 7,636	2,062 ± 315	1,457 ± 644

[a] MLCs were performed between family members and controls in muleiple combinations of responders to stimulators. In addition to measuring the response at 6 days ("MLC response" column), supernatants were removed from each combination, sterilized and assayed for MLC-MF activity on resting cells ("MLC-MF assay" column) from two individuals (responders 1 and 2).

responses measured in 32 different MLC combinations (MLC response column) and the MLC-MF activity of the supernatants from those MLCs, as tested on resting MNL from two responders. A pattern of associations appears such that high MLC cultures possessed high MLC-MF activity in the supernatant and conversely, low MLCs had low MLC-MF present.

Their association was directly tested for statistical significance for both responders (Figures 13,14). In both cases, correlation was established between the MLC response and MLC-MF activity. This indicates that the degree of blastogenesis seen in the MLC may be the result of the level of MLC-MF produced, which may differ from one person to the next, or alternately, the level of MLC-MF may be the result of the degree of blastogenesis. In any case, an association between the two appears to exist.

Titration of MLC-MF Activity

A MLC supernatant known to possess good MLC-MF activity was serially diluted to determine at what dilution no response would occur. As indicated in Figure 15, this particular MLC supernatant induced significant mitogenic doses to a 1:32 dilution. In general, most MLC-MF assays were run at a 1:2 dilution.

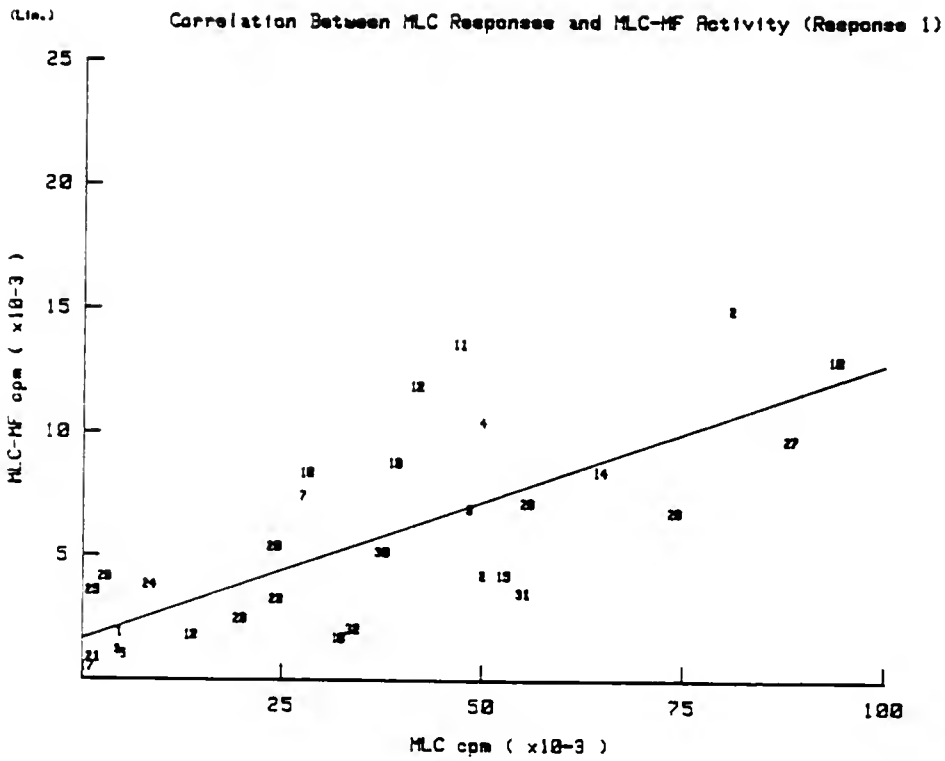
Time Course Response of Resting and PLT Cells to MLC-MF

A series of experiments were designed to determine when the peak response occurred to MLC-MF by resting cells and 10 day old MLC-primed (PLT) cells. Figure 16 shows that both cell populations respond to MLC-MF with the same kinetics as they do to alloantigen stimulation. Resting cells, in the example shown, had the peak proliferation to MLC-MF at 6 days, the same peak as seen in the MLC. An analogous situation

Figure 13. Statistical analysis of the correlation between MLC responses and the MLC-MF activity of the supernatants obtained from those MLCs (all values obtained from Table 6). Figure 13 represents the MLC cpm versus MLC-MF cpm as detected on responder 1. Each number on the line represents the combination number described in Table 6. There was significant correlation since the linear coefficient was significantly different from 0 with P = .0000027.

Correl. coeff. (r)	.72
Sample size (n)	32.00
t statistic	5.76
Degr. freedom	30.00

	one-sided test	two-sided test
p value	.0000014	.0000027
confidence level (%)	99.9999	99.9997



[illegible]	.78		
Sample size n	32.00		
t statistic	5.35		
Degr. freedom	30.00		
		one-sided test	two-sided test
p value		.0000043	.0000086
Confidence level (%)		99.9996	99.9991

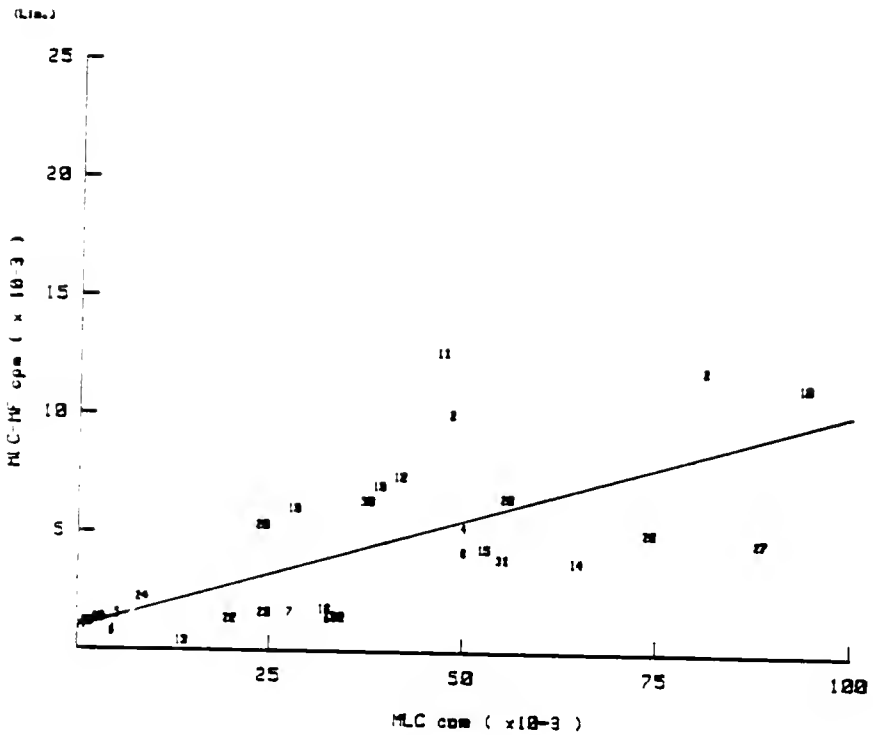


Figure 14. Statistical analysis of the correlation between MLC responses and the MLC-MF activity of the supernatants obtained from those MLCs (all values obtained from Table 6). Figure 14 represents the MLC cpm versus MLC-MF cpm as detected on responder 2. Each number on the line represents the combination number described in Table 6. There was significant correlation since the linear coefficient was significantly different from 0 with P = .0000086.

Figure 15. Titration of MLC supernatant. MLC-MF activity (as detected on resting MNL) of serial dilutions of an MLC supernatant. Each point is the mean of triplicate values $\pm$ the S.D.

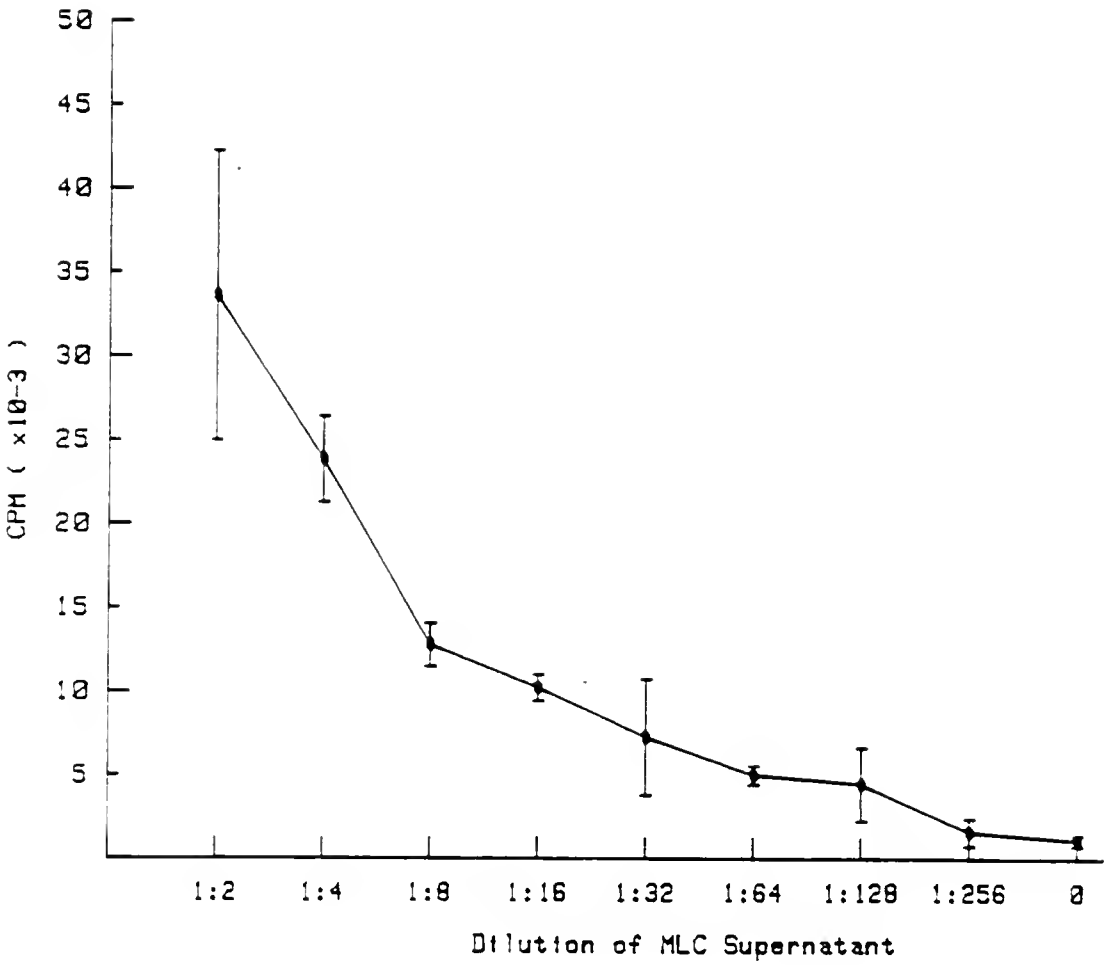
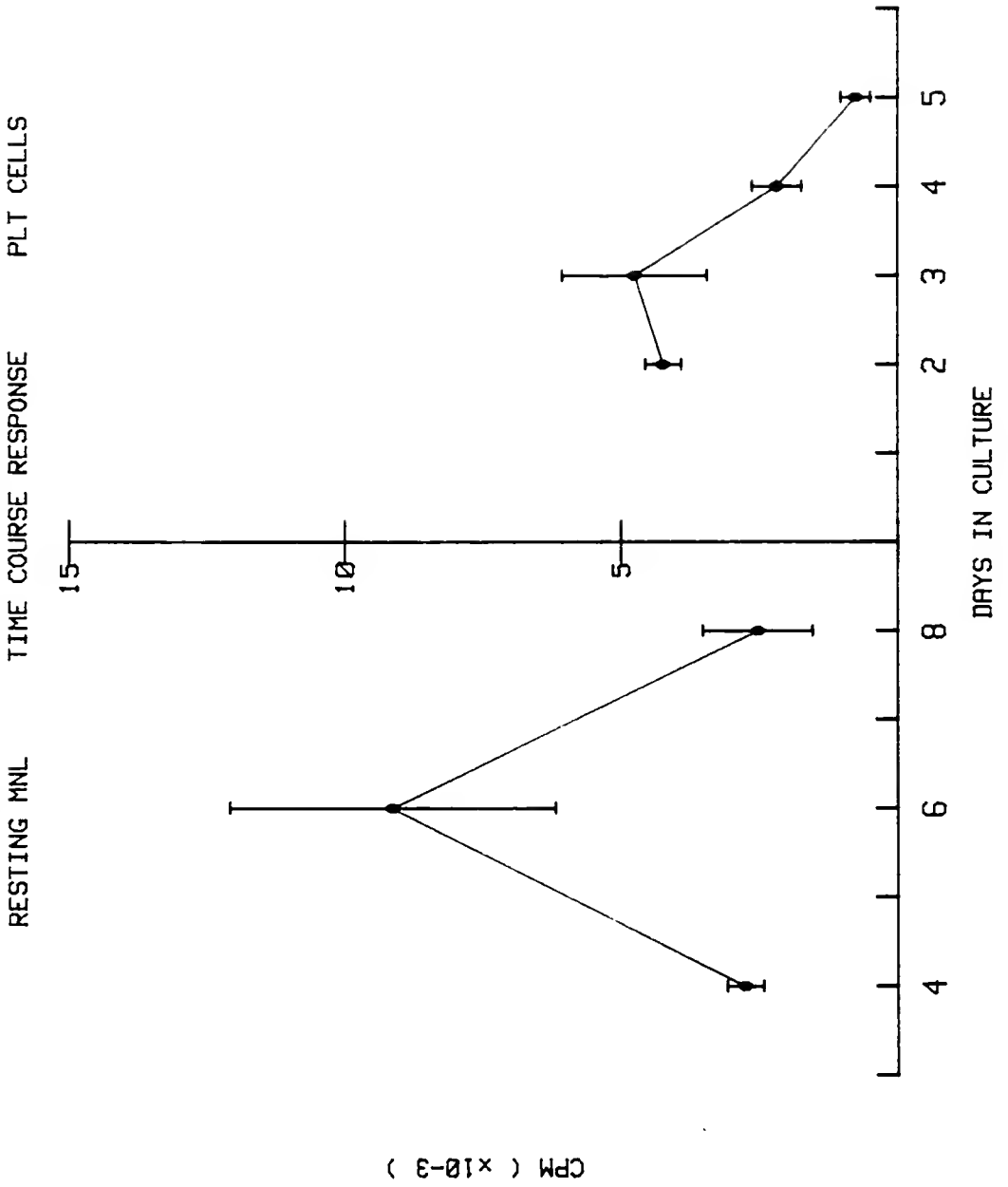

50
45
40
35
30
25
20
15
10
5
CPM (x10-3)
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
0
Dilution of MLC Supernatant

Figure 16. The time course response of resting MNL and PLT cells to MLC supernatants. The left side of Figure 16 shows the response by resting MNL to MLC-MF when measured at 4, 6 and 8 days. The right side of this figure shows the optimal day of response to MLC-MF by MLC-primed or PLT cells. The points represent the mean of triplicate values $\pm$ the S.D.


RESTING MNL TIME COURSE RESPONSE PLT CELLS
15
10
5
CPM (x10-3)
4
6
8
2
3
4
5
DAYS IN CULTURE

exists with PLT cells. These memory cells respond with secondary kinetics (2-3 days) if exposed to the same alloantigen which was used for the primary stimulation. Similarly, culture supernatants with MLC-MF activity induced a maximal proliferation 2-3 days following the initiation of the incubation.

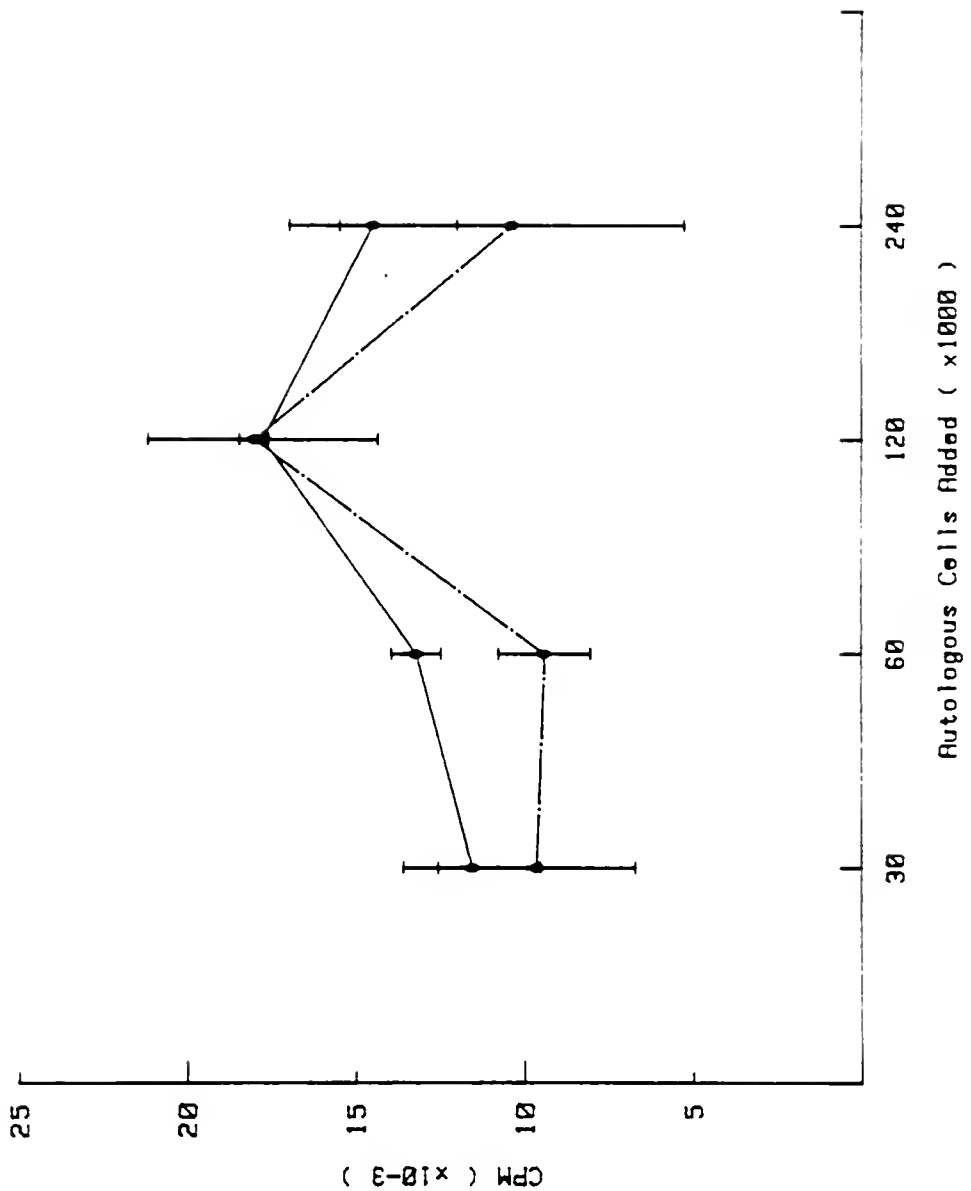
Enhancement of the Response to MLC-MF by Irradiated Autologous Cells

As mentioned before, the MLC-MF activity was found to enhance the autologous MLC (AAx) combinations but not the allogeneic MLC. The role of the irradiated cells appeared important since the response of A alone to MLC-MF was much lower than the AAx combination (an example is shown in Figure 19). A study was done to determine the optimal number of irradiated cells which was needed by incubating 30 X 10^3 MNL with varying concentrations of autologous irradiated cells (Figure 17). In addition, the same number of non-irradiated, autologous cells were added to other wells to compare the responses. As indicated in Figure 17, wells which were given non-irradiated autologous cells produced slightly higher responses than those given irradiated cells at the lower concentrations. However, when 120 X 10^3 irradiated cells were added to the 30 X 10^3 MNL the response was equal to that of 150 X 10^3 MNL. Since the irradiated cells themselves were not proliferating to the MLC-MF, it was clear that they were augmenting or "helping" the response of the non-irradiated MNL.

Determination of the Optimal Radiation Dose for the "Helper" Effect by Autologous Cells

The observations that irradiated cells were enhancing the response to MLC-MF prompted experiments designed to determine whether the dose

Figure 17. Helper effect of non-irradiated and irradiated autologous cells. Graded numbers of non-irradiated (—) and irradiated (—·—) MNL were added to 30 X 10^3 autologous MNL (responders) in the presence of MLC-MF containing supernatant. Assay performed is the MLC-MF assay as described in Materials and Methods. The points represent the mean of triplicate values ± the S.D. The response of the 30 X 10^3 MNL with no added cells was 9,602 ± 1,702.


CPM (x10-3)
25
20
15
10
5
30
60
120
240
Autologous Cells Added (x1000)

of irradiation used until then (3000 rads) was the optimal dose for the helper effect. Cells were exposed to a series of radiation doses and then added at a concentration of 120 X 10^3 cells to 30 X 10^3 autologous, non-irradiated cells (responders) in the presence of 50% MLC-MF. The response measured at 6 days (Figure 18) indicates that the optimal radiation dosage for helper activity occurred from 2600 to 3200 rads. These cells alone did not respond to MLC-MF.

Using these experiments as a basis, all MLC-MF assays done in which autologous irradiated cells were added to the 30 X 10^3 responders, used a "helper" concentration of 120 X 10^3 cells given 3000 rads.

Adherent Cells Provide the "Helper" Effect in the Irradiated Cell Population

The observation that 120 X 10^3 unseparated, irradiated MNL augmented the response of 30 X 10^3 autologous MNL allowed us to study the role that monocytes played in the response to MLC-MF. The first pair of bars in Figure 19 represents the response with and without MLC-MF of 30 X 10^3 nonadherent MNL. This cell population contained a very small percentage (3% by electronic sizing analysis [ESA] and 0% by peroxidase) (Figure 20) of monocytes, in comparison to the unseparated MNL population (23% by ESA and 10% by peroxidase). The addition of 120 X 10^3 irradiated, unseparated MNL significantly enhanced the response to MLC-MF. In contrast, the addition of the same number of irradiated nonadherent cells did not affect the response. A repeat of this experiment is shown in Figure 21 (Coulter analysis in Figure 22) with similar results.

Figure 18. Effect of varying radiation doses on helper activity. Autologous helper cells (120×10^3) given varying radiation doses were added to autologous responder cells (30×10^3) in the MLC-MF assay. Each point represents the mean of triplicate values $\pm$ the standard deviation.

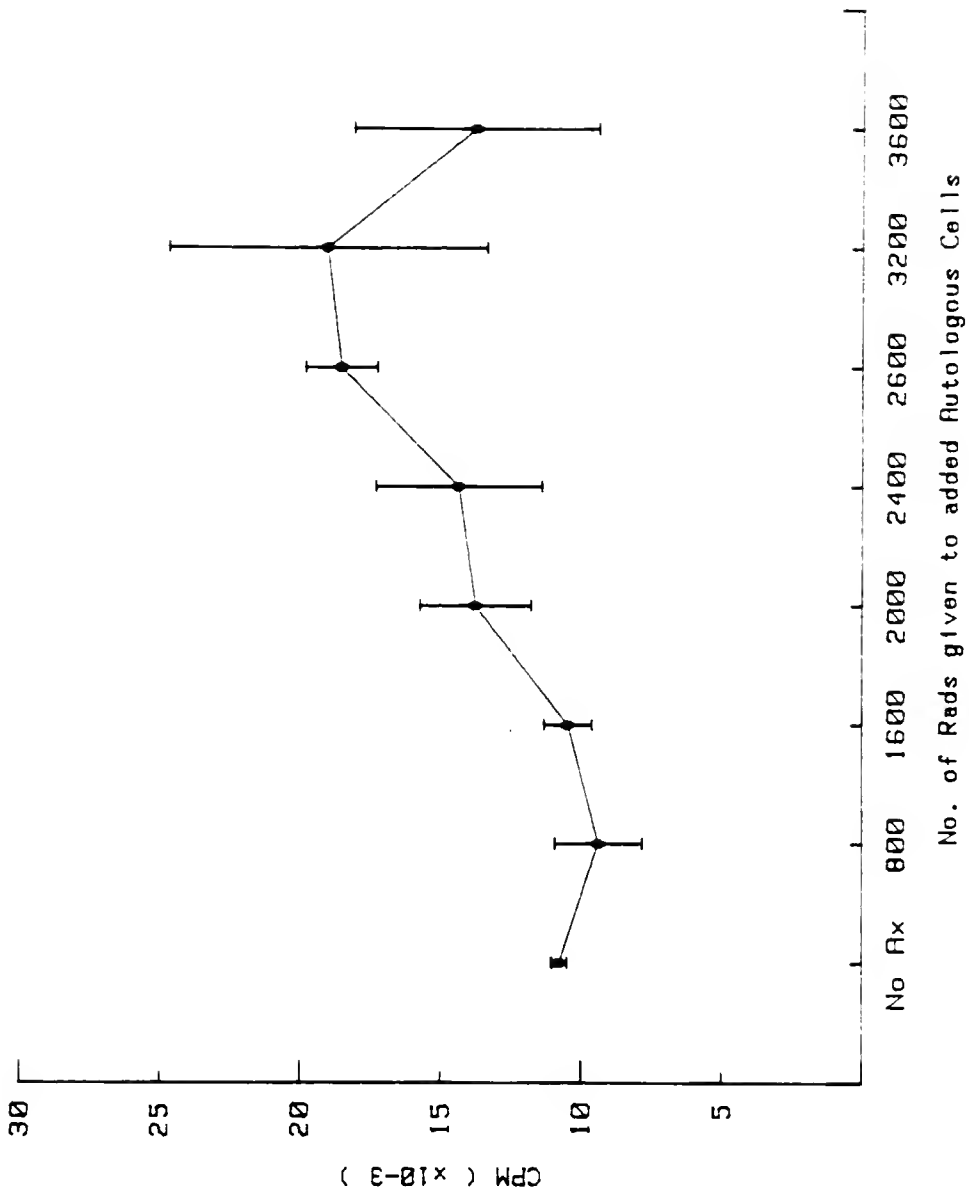

30
25
20
15
10
5
CPM (x10-3)
No Rx
800
1600
2000
2400
2600
3200
3600
No. of Rads given to added Autologous Cells

Figure 19. Determination of the cells providing the "help" in the irradiated MNL population. Open bars represent wells with no MLC-MF and striped bars are wells with 50% MLC-MF. Ax(120) corresponds to 120 X 10^3 autologous responders. Anax(120) corresponds to nonadherent, irradiated MNL which were added to responders. The first pair of bars had only responders, with no autologous helpers added. The bars represent the mean of triplicate values $\pm$ the S.D.

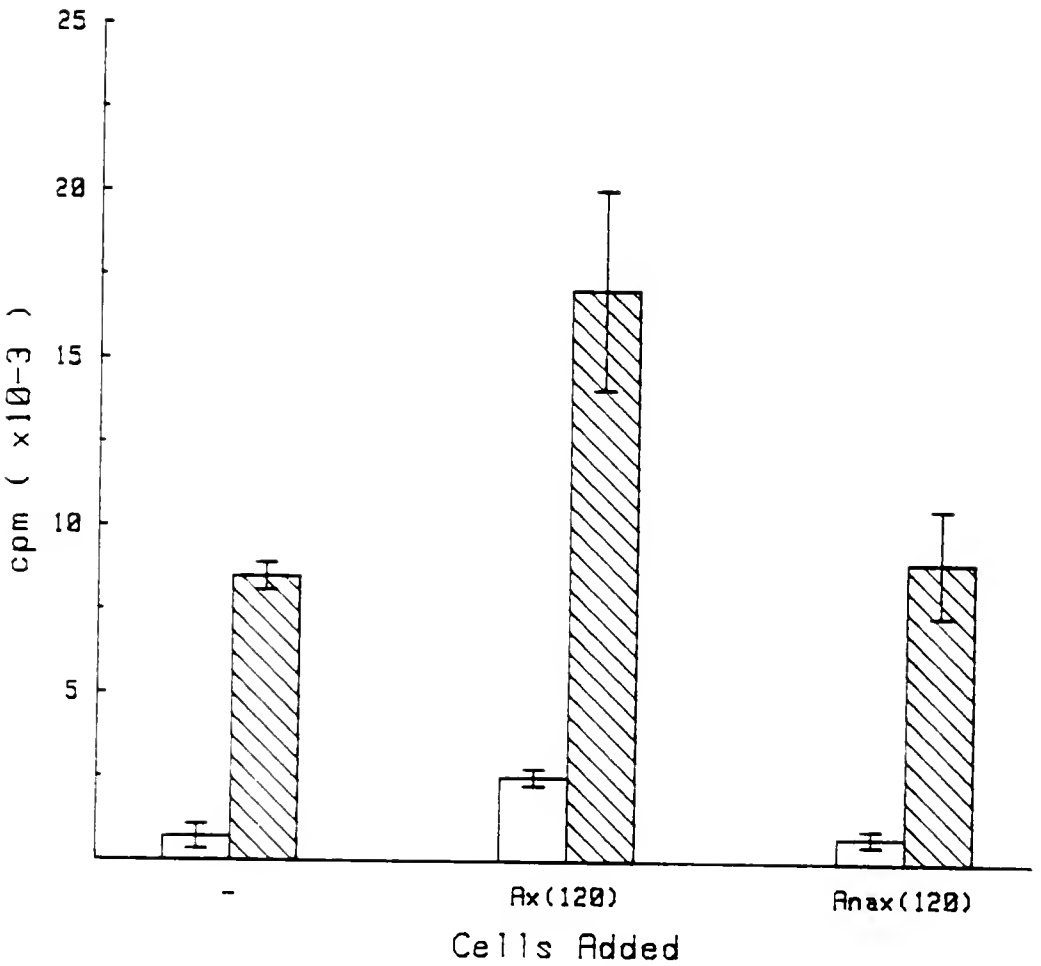
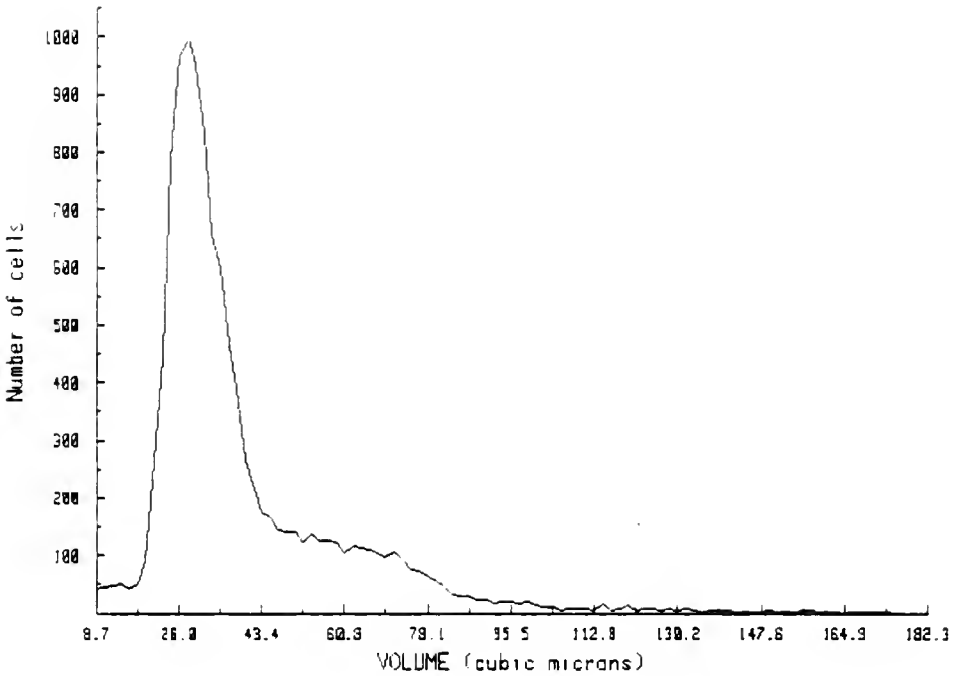

25
20
15
10
5
cpm (x10-3)
-
Ax(120)
Anax(120)
Cells Added

Figure 20. Coulter analysis of irradiated unseparated and NA MNL that were used as helpers in Figure 19. Other data on these cells included: Unseparated MNL had 8% B cells (Surface IgM), 10% peroxidase positive cells and 23% "monocytes" by Coulter analysis (top of Fig. 20). NA MNL had 1% B cells (Surface IgM), 0% peroxidase positive cells and 3% "monocytes" by Coulter analysis (bottom of Fig. 20).


Number of cells
1000
900
800
700
600
500
400
300
200
100
8.7
26.0
43.4
60.8
78.1
95.5
112.8
130.2
147.6
164.9
182.3
VOLUME (cubic microns)

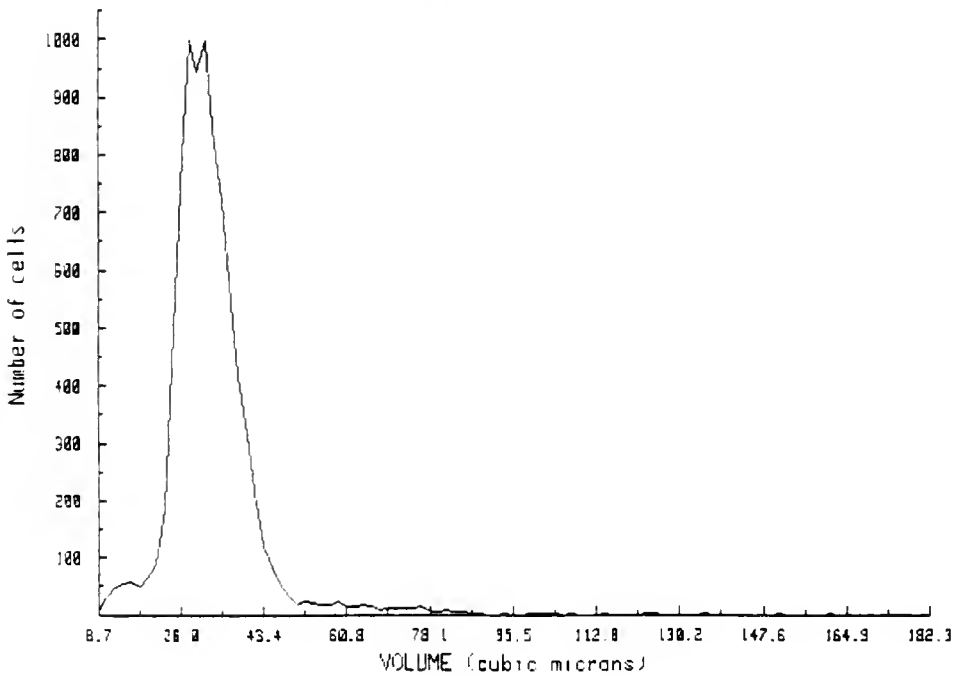

Number of cells
1000
900
800
700
600
500
400
300
200
100
8.7
26.0
43.4
60.8
78.1
95.5
112.8
130.2
147.6
164.9
182.3
VOLUME (cubic microns)

Figure 21. Determination of the cells providing the "help" in the irradiated MNL population. Open bars represent wells with no MLC-MF and striped bars are wells with 50% MLC-MF. Ax(120) corresponds to 120 X 10^3 autologous responders. Anax(120) corresponds to nonadherent, irradiated MNL which were added to responders. The first pair of bars had only responders, with no autologous helpers added. The bars represent the mean of triplicate values $\pm$ the S.D.

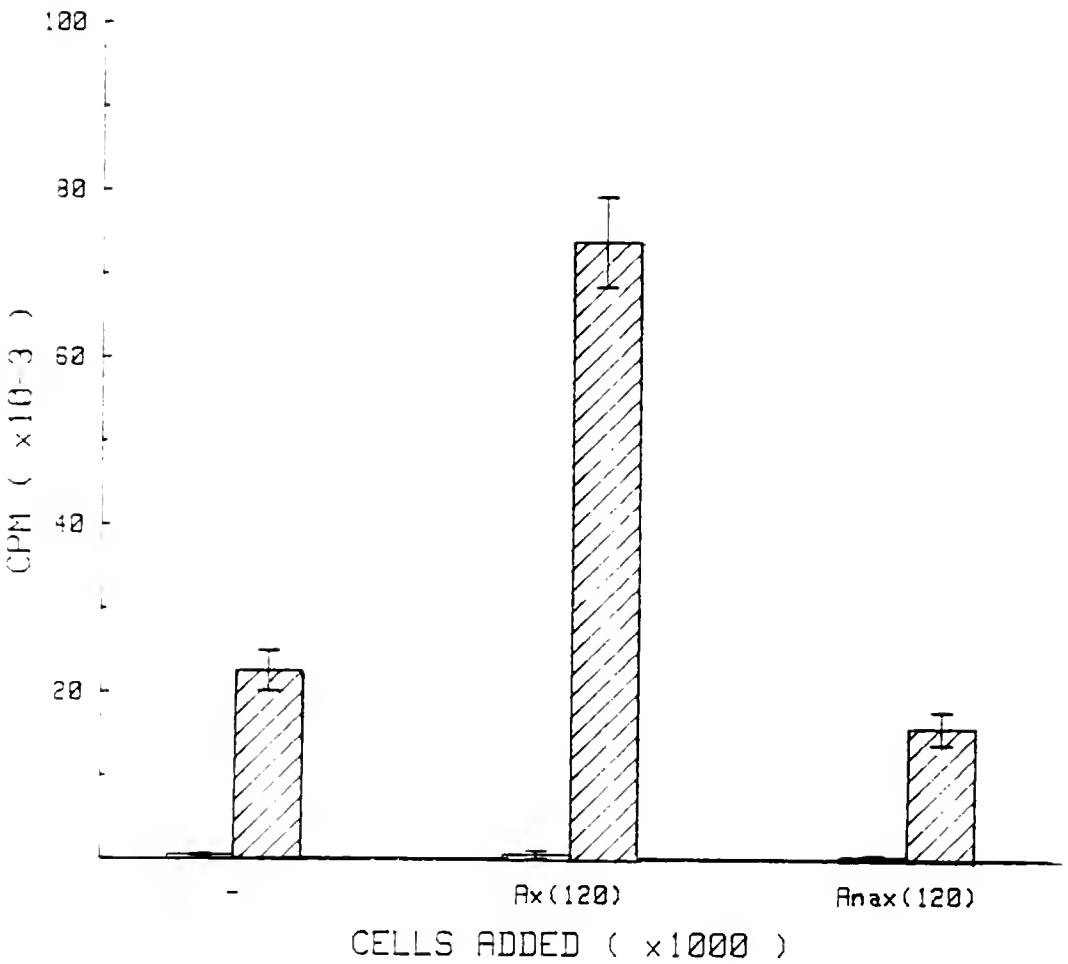
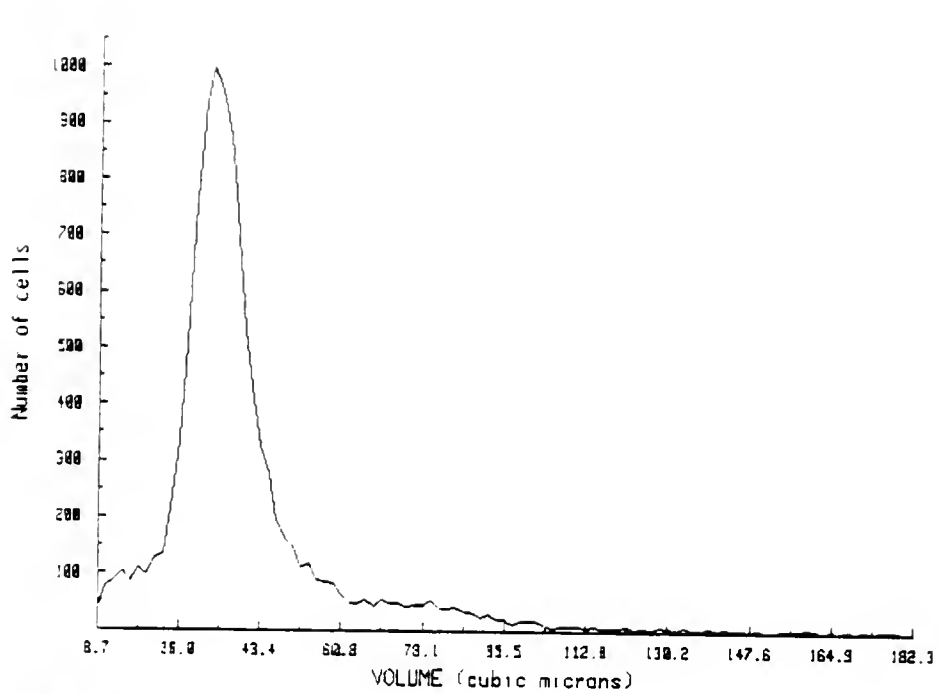
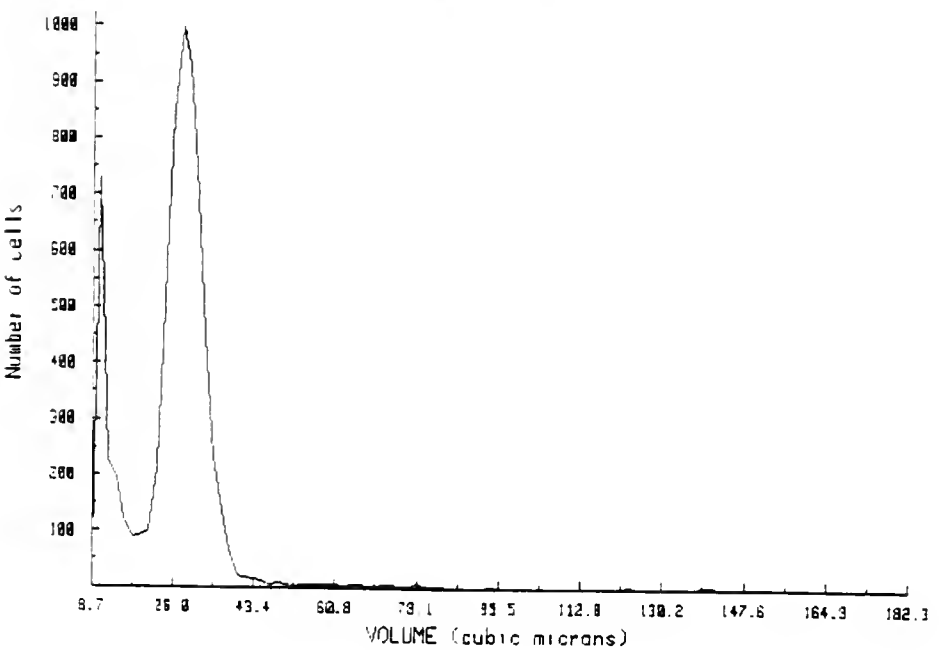

100
80
60
40
20
CPM (x10-3)
-
Ax(120)
Anax(120)
CELLS ADDED (x1000)

Figure 22. Coulter analysis of irradiated unseparated and NA MNL used as helpers in Figure 21. Unseparated MNL contained 7% monocytes by Coulter analysis and NA MNL contained 1% monocytes by Coulter analysis.


Number of cells
VOLUME (cubic microns)


Number of cells
VOLUME (cubic microns)

These results suggest that monocytes amplify the response to MLC-MF although they may not be required to induce it. The possibility is minimal that B cells were providing the help since in data not shown, nonadherent cells from petri dishes, which contain many B cells and few monocytes, provided little help when added to nonadherent responders.

Proliferation to MLC-MF is not Due to Additional MF Production

Since IL1, a monocyte product, can induce lymphocytes to produce IL2 (171), it was next investigated whether the MF activity of MLC supernatants was due to a factor directly mitogenic to MNL or to a factor only capable of inducing MF production by the responding cells, but not being mitogenic itself.

Two approaches were utilized to answer this question. The first approach involved the use of adrenal steroids, which have been reported to inhibit IL2 production but not its effect on activated lymphocytes (169). A representative experiment is described in Table 7. MLC-MF production was induced in the presence and in the absence of hydrocortisone. A significant inhibition of MLC-MF production was seen at concentrations of 10^{-5} and 10^{-6} M. However, when hydrocortisone was added after the MF was produced, the effect on responder cells was minimal. Thus, since production of MLC-MF does not occur in the presence of hydrocortisone, the mitogenic activity observed when hydrocortisone is added to MLC-MF cannot be explained by the production of additional MF by the responder or irradiated cells.

A second, more direct approach was also utilized to explore the same problem. MLC-MF was incubated for 2 days at 37°C alone and in the presence of unseparated MNL (Table 8). These supernatants, when tested

TABLE 7

EFFECT OF HYDROCORTISONE ON THE GENERATION AND EFFECT OF MLC-MF

HC Present During Generation of MLC-MF[a]	HC Added to MLC-MF[b]	cpm[c] Exp 1	Exp 2
0	0	12,285 ± 1,771[d]	14,538 ± 3,109
10^{-5}M	0	1,226 ± 120	2,259 ± 665
10^{-6}M	0	4,707	4,415 ± 537
0	10^{-5}M	9,268 ± 2,568	7,772 ± 3,525
0	10^{-6}M	12,601 ± 4,588	10,722 ± 3,420
No MLC-MF Added		2,937 ± 1,705	2,044 ± 788

(a) HC was added at concentrations of 10^{-5}M and 10^{-6}M at the start of the production of MLC-MF in several tubes.

(b) HC was added at the start of the MLC-MF assay to MLC-MF produced in absence of HC.

(c) Counts per minute (mean of triplicate values ± S.D.).

(d) All supernatants were tested in two MLC-MF assays (described in Materials and Methods).

TABLE 8

LACK OF ADDITIONAL ACTIVITY IN MLC-MF PREINCUBATED WITH IRRADIATED CELLS[a]

EXPERIMENT 1

Supernatant	MLC-MF Activity (cpm + S.D.)
MLC sup. without preincubation	38,619 ± 4,143
MLC sup. incubated alone for 2 days	46,727 ± 4,451
MLC sup. incubated with Ax(120) for 2 days	15,963 ± 4,191

(a) Supernatants containing MLC-MF activity were incubated for two days at 37°C with or without irradiated, unseparated MNL. In Figure 23 (Expt.2), adherent, irradiated MNL were also used to preincubate with MLC-MF. Following this period, the supernatants were collected, spun down and tested for their MLC-MF activity (in the MLC-MF assay) in comparison to fresh MLC supernatant from the same lot.

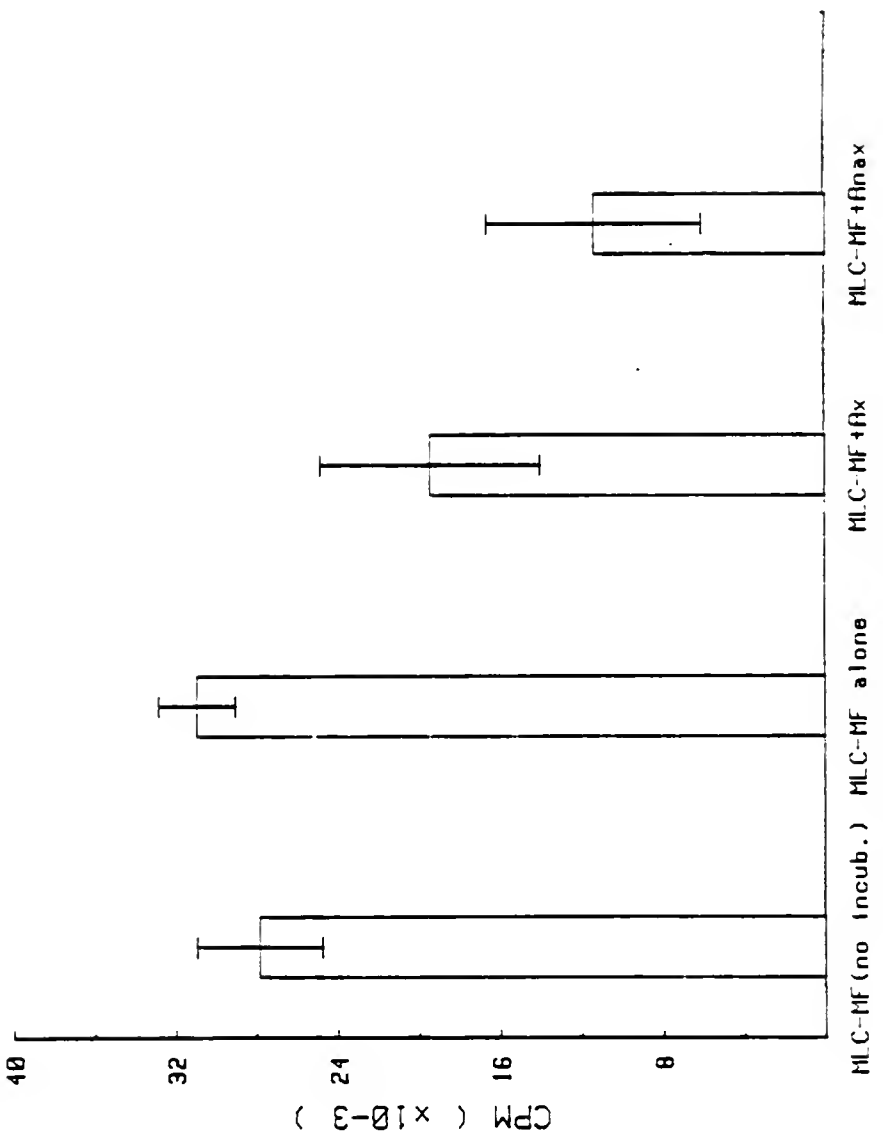
for their MLC-MF activity after the incubation period, showed that the supernatants incubated with the irradiated cells actually possessed less and not greater MF activity in comparison to the other supernatants incubated in cell-free conditions. Figure 23 shows a repeat of this experiment with the added combination of supernatant incubated with nonadherent MNL for two days. Again supernatants collected after two days in the presence of irradiated unseparated and nonadherent MNL, showed less MF activity than the controls. These results argue against the possibility that additional MF is produced by irradiated cells in the presence of MLC-MF. The "helper" effect provided by the monocytes thus appears to be through a direct interaction with the responder cells and the MLC-MF, rather than through the release of mitogenic factors.

Role of Putative Soluble Alloantigens

The next experiments addressed the question of whether the MLC-MF preparation contained any antigen that might be involved in the induction of proliferation. Since antigens in the HLA-D region are thought to be the primary stimuli in the MLC, it was reasonable to assume that they could be involved in this system.

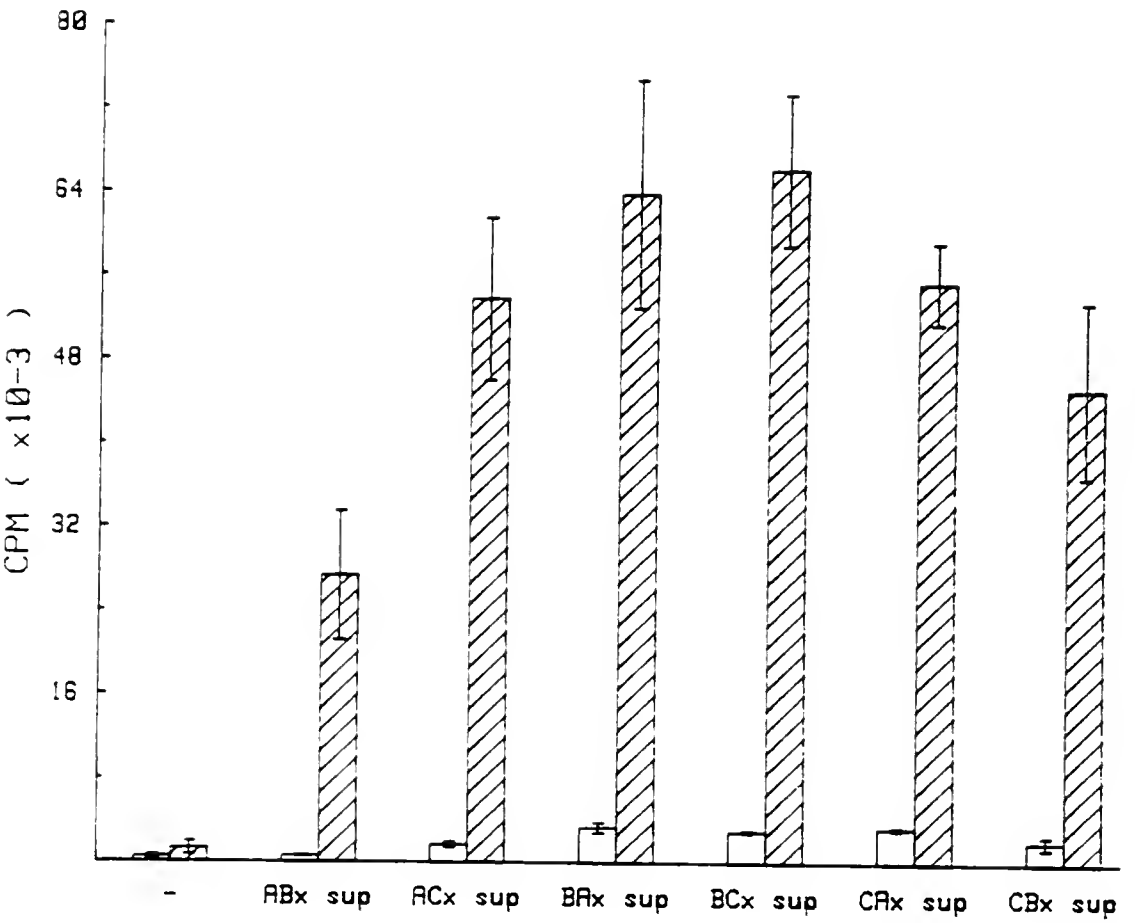
Experiments were designed in which MLC-MF was generated in all possible MLC combinations between three unrelated individuals A,B and C (A vs. B, A vs. C, B vs. C). Each MLC-MF preparation was then tested on each individual to determine whether a genetically restricted response to MLC-MF existed. The results in Figure 24, 25 and 26 indicate that no restriction was found in the response to MLC-MF since each person showed no preferential reactivity to any supernatants. It is also evident that

Figure 23. Effect of preincubation on MLC-MF activity (Experiment 2). Supernatants containing MLC-MF activity were incubated for two days at 37°C with or without irradiated MNL (either unseparated or adherent). Following this period, the supernatants were collected, spun down and tested for their MLC-MF activity (in the MLC-MF assay) in comparison to fresh MLC supernatant from the same lot.



Combinations Incubated for Two Days

Figure 24. Lack of genetic restriction in the response to MLC-MF. Supernatants from MLCs (in every combination) between three normal, unrelated individuals (A, B and C) were collected and tested on fresh responder MNL from individual A. The open bars are the responders (30×10^3) plus the supernatants while the striped bars are responders, autologous irradiated MNL (120×10^3) and supernatants. All bars represent the mean of triplicate values $\pm$ the S.D.


80
64
48
32
16
CPM (x10-3)
-
ABx sup
ACx sup
BAx sup
BCx sup
CAx sup
CBx sup

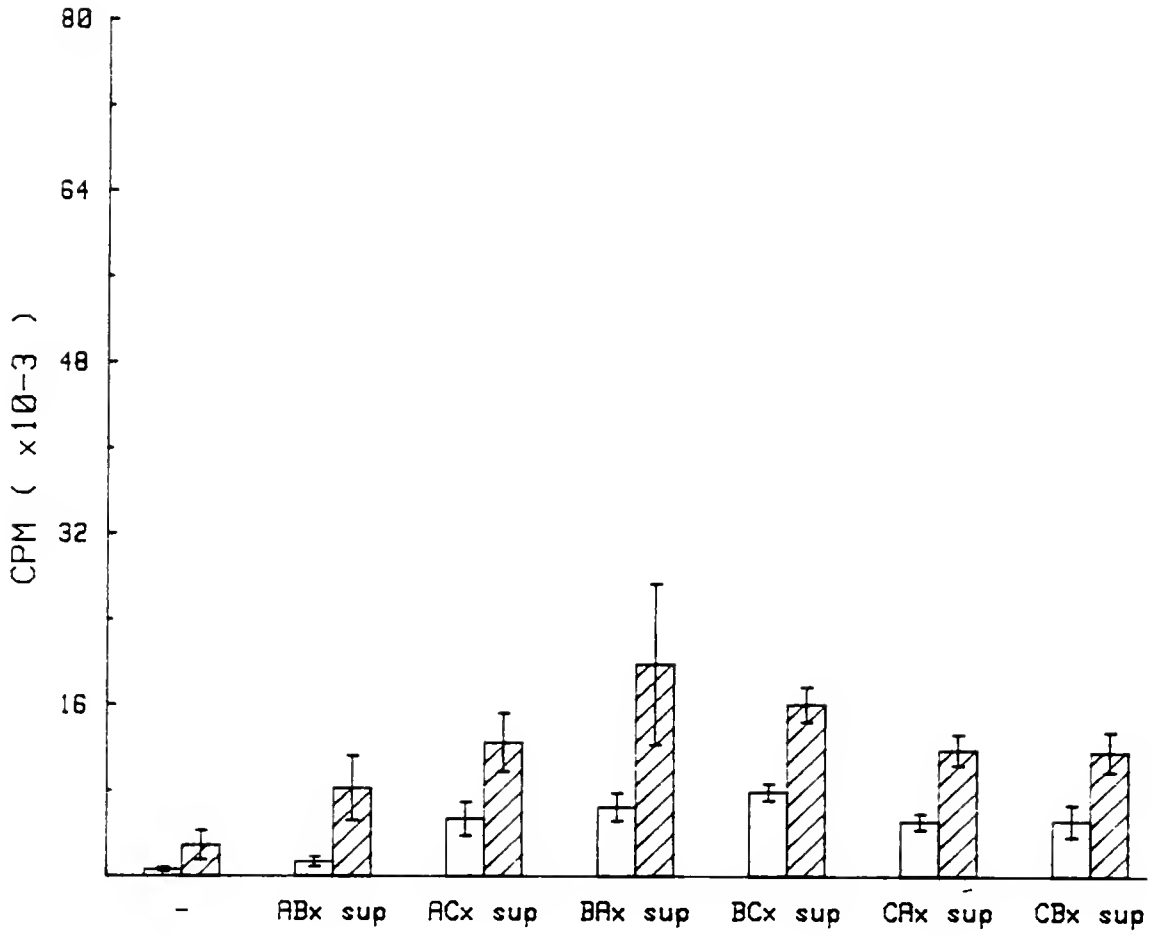


Figure 25. Lack of genetic restriction in the response to MLC-MF. Supernatants from MLCs (in every combination) between three normal, unrelated individuals (A, B and C) were collected and tested on fresh responder MNL from individual B. The open bars are the responders (30×10^3) plus the supernatants while the striped bars are responders, autologous irradiated MNL (120×10^3) and supernatants. All bars represent the mean of triplicate values $\pm$ the S.D.

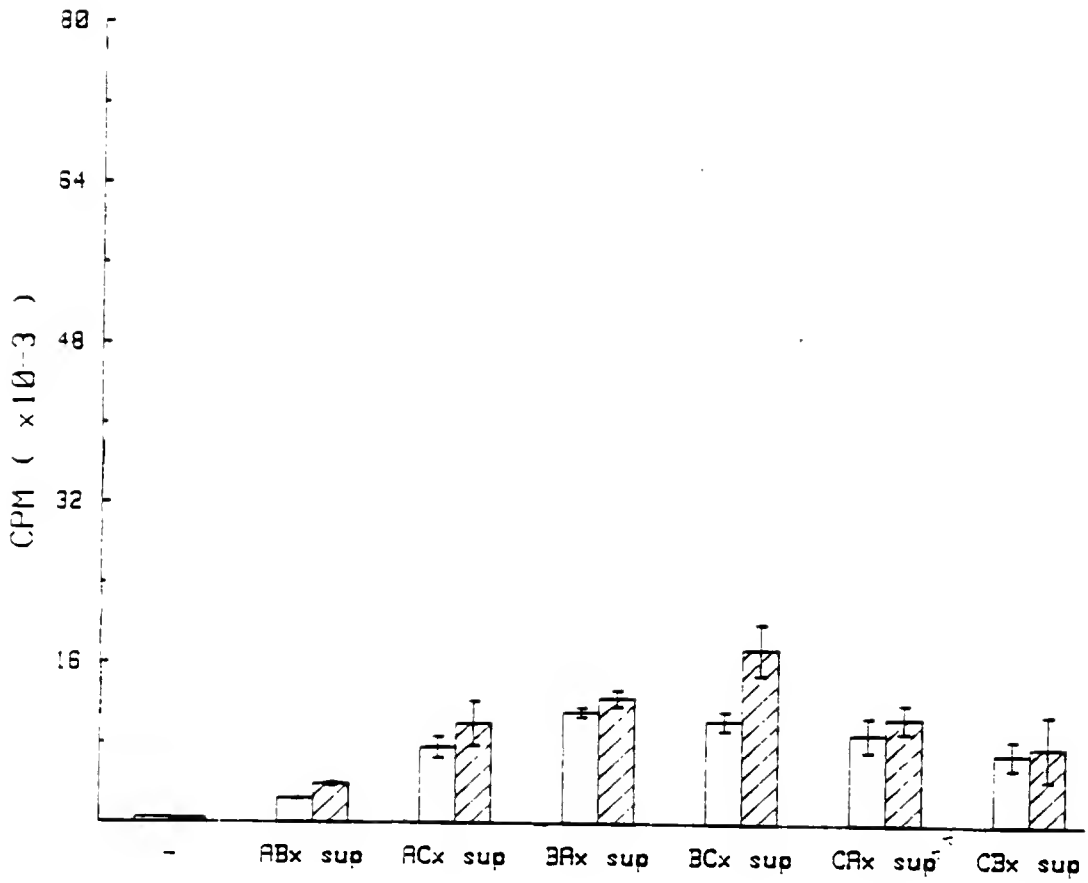


Figure 26. Lack of genetic restriction in the response to MLC-MF. Supernatants from MLCs (in every combination) between three normal unrelated individuals (A, B and C) were collected and tested on fresh responder MNL from individual C. The open bars are the responders (30×10^3) plus the supernatants while the striped bars are responders, autologous irradiated MNL (120×10^3) and supernatants. All bars represent the mean of triplicate values $\pm$ the S.D.

individual A is a high responder to all the supernatants in comparison to the other two individuals. In addition, some MLC combinations induced supernatants which obviously possessed higher activity with all three responders tested. Thus although no specificity exists in the response to MLC-MF, variability does exist in the overall responsiveness of an individual and in the levels of MLC-MF induced from one MLC to the next.

The above results, which suggested a lack of involvement of alloantigens, were strongly sustained in other experiments. MLC-MF was produced and tested in a mother-child combination in which the mother behaved as HLA-D homozygous. In repeated MLCs the child responded poorly to the mother but the mother responded well to the child. MLC-MF production was induced with equal numbers of irradiated cells from the child and non-irradiated cells from the mother. If alloantigens present in the preparations were necessary for MF action, the supernatant would be stimulatory to the mother's responder cells since she had reacted in MLC to her child. Conversely, if alloantigens were necessary for MF activity, little or no response would be induced in the child's cells because he did not respond to his mother's antigens. Table 9 shows that this was not the case. Cells from both the mother and the child showed comparable responses to the MLC-MF generated by the mixtures of both cells.

Response of MNL (To MLC-MF) Treated with OKT Antibodies

The fact that T cell enriched MNL (Figure 19) proliferated to MLC-MF indicated that the responding population were indeed T cells (although B cells could also be proliferating). In an effort to identify the T cell subpopulation which was responding, T cell subsets were depleted from MNL populations by treatment with OKT antibodies (which identify human

TABLE 9

MLC AND MLC-MF RESPONSE OF A HOMOZYGOUS HLA-D INDIVIDUAL AND HER CHILD

Responder	HLA-DR	MLC-MF Generated By BAx MLC	Control MLC-MF	MLCs (Stimulators) Ax	Bx	Cx
(A) Child	4,7	8,340 ± 1,488[a]	19,094 ± 3,356	2,341 ± 281	7,601 ± 1,997	44,962 ± 459
(B) Mother	4,-	5,131 ± 1,768	11,538 ± 786	22,751 ± 3,485	3,241 ± 239	43,107 ± 541
(C) Control		10,065 ± 1,357	51,829 ± 5,981	103,678 ± 29,644	N.D.[b]	4,016 ± 203

(a) Counts per minute (means of triplicate cultures) ± S.D.

(b) Not done. This variable was accidentally lost in this MLC. However Bx cells proved to be excellent stimulators in two other MLCs against three unrelated individuals.

T cell subsets) and complement and the remaining cells were tested for their reactivity to MLC-MF and in MLC (Table 10). While the OKT4 population seems to be the one mainly involved in the MLC response, depletion of these cells did not affect the response to MLC-MF.

Column Chromatography of MLC-MF

Activities on human cells. Supernatants from MLCs were concentrated and run through G100 molecular seive chromatography to determine whether MLC-MF activity could be localized to any particular fractions. Figure 27 shows that MLC-MF was found to have a discrete peak of activity (frac. 28-37) when all fractions were assayed on resting MNL (AAx). The fractions containing the MLC-MF activity came out between the ovalbumin (45,000 mw) marker (peak at fraction 29) and the lysozyme (14,500 mw) marker (peak at fraction 46). Thus, in this run, MLC-MF was estimated to have an approximate molecular weight of 30,000. The void volume was always at fraction 15. The fractions were also tested on a long term culture human line which was only responsive to TCGF. The results (Figure 28) show several peaks of activity with the highest peak correlating directly with the MLC-MF peak. A comparison of the two activities is shown in Figure 29. Finally, when fractions were tested for their mitogenic activity on human thymocytes, little activity was noted (not shown).

Activities on mouse cells. In an effort to determine the costimulator activity present in the column, fractions were assayed in the standard costimulator assay using mouse thymocytes in the presence of a submitogenic dose of ConA. An example of the costimulator activity of MLC and PLT supernatants is shown in Table 11. An analysis of the

TABLE 10

RESPONSE OF MNL TREATED WITH OKT4 AND OKT8 MONOCLONAL ANTIBODIES[a]

EXPERIMENT 1

Responder (Nonadherent MNL)	—	MLC-MF	Ax(120)+[b] MLC-MF	Bx(120)[c]
A(30) (Untreated)	816 ± 80	6,681 ± 928	7,493 ± 2,027	60,678 ± 1,518
A(30) (OKT4 and Complement)	733 ± 97	6,557 ± 190	10,943 ± 712	10,030 ± 2,698
A(30) (OKT8 and Complement)	1,272 ± 266	8,292 ± 1,293	5,942 ± 1,995	68,267 ± 4,642
A(30) (Complement)	1,260 ± 582	10,227 ± 1,158	11,364 ± 3,487	59,969 ± 3,379

EXPERIMENT 2

Responder (Unseparated MNL)	—	MLC-MF	Ax(120)+ MLC-MF	Bx(120)
A(30) (Untreated)	392 ± 119	2,834 ± 675	5,150 ± 756	114,000 ± 5,052
A(30) (OKT4 and Complement)	1,579 ± 720	N.D.[d]	5,145 ± 1,681	10,251 ± 2,645
A(30) (OKT8 and Complement)	2,613 ± 1,005	N.D.	5,485 ± 263	133,782 ± 5,050
A(30) (Complement)	420 ± 248	N.D.	5,310 ± 919	126,917 ± 4,481

(a) The cells used for treatment with OKT4 and OKT8 antibodies were nonadherent MNL in Experiment 1 and unseparated MNL in Experiment 2. Treatment with the antibodies and complement is described in Materials and Methods. Following the treatment the cells were counted and 30 X 10^3 viable cells were added to the wells as responders. All numbers in parenthesis are X 10^3.

(b) Unseparated irradiated MNL were used as the source of Ax in both Experiments.

(c) An unrelated individual was used as the source of Bx for stimulators in MLC.

(d) Not done.

Figure 27. Assay of column fractions for MLC-MF activity. Column fractions from a Sephadex G-100 separated MLC supernatant were individually tested in the MLC-MF assay (30×10^3 responder MNL plus 120×10^3 irradiated, autologous MNL). All fractions were tested at 50% concentrations. Unfractionated MLC supernatant had $14,986 \pm 721$ cpm.

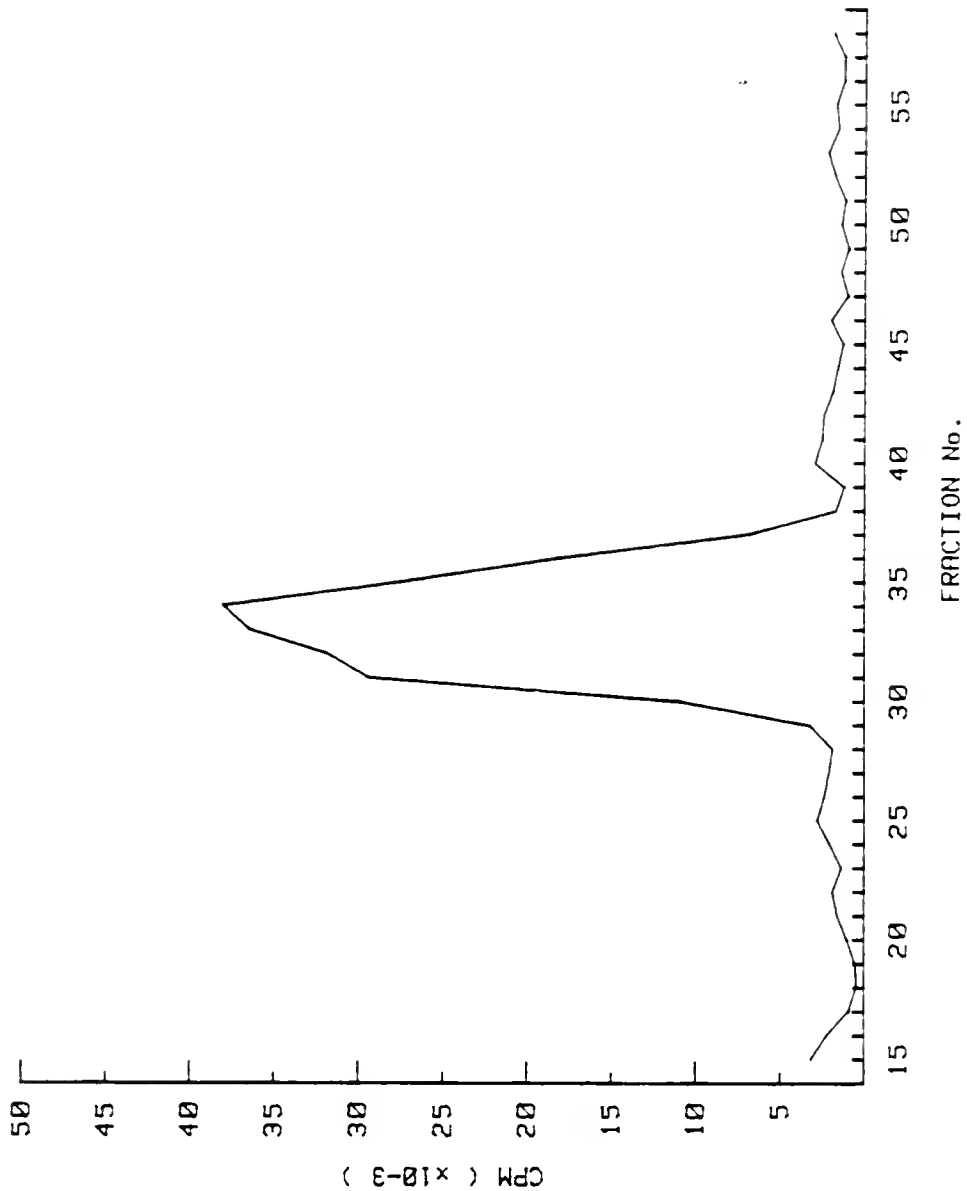

CPM (x10-3)
50
45
40
35
30
25
20
15
10
5
15
20
25
30
35
40
45
50
55
FRACTION No.

Figure 28. Assay of column fractions for TCGF activity. Column fractions from a Sephadex G-100 separated MLC supernatant were individually tested on the human long term T cell line, CTCL, to determine levels of human TCGF. All fractions were tested at 50% concentration. Unfractionated MLC supernatant had 2,542 $\pm$ 274 cpm.

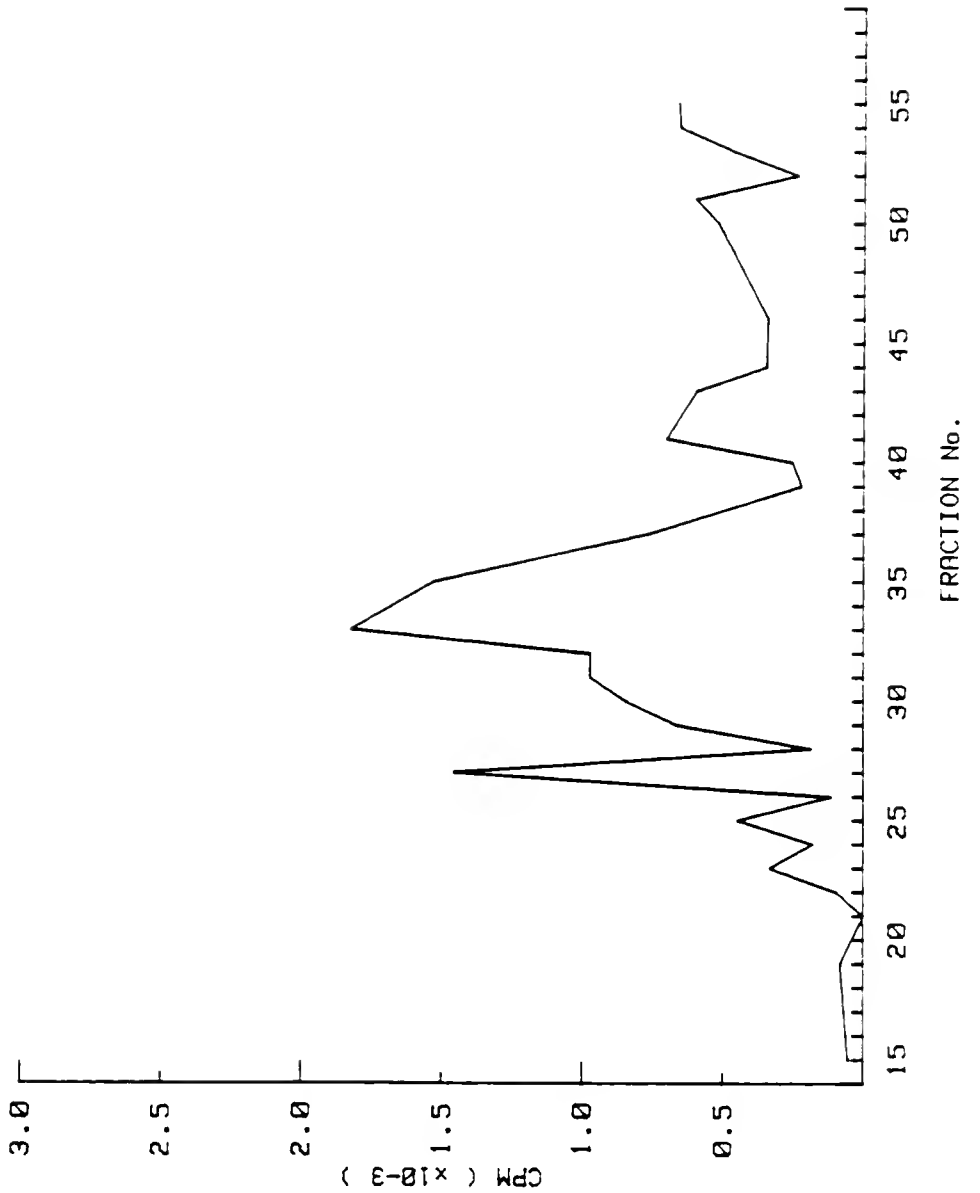

3.0
2.5
2.0
1.5
1.0
0.5
CPM (x10-3)
15
20
25
30
35
40
45
50
55
FRACTION No.

Figure 29. A comparison of Figures 27 and 28.

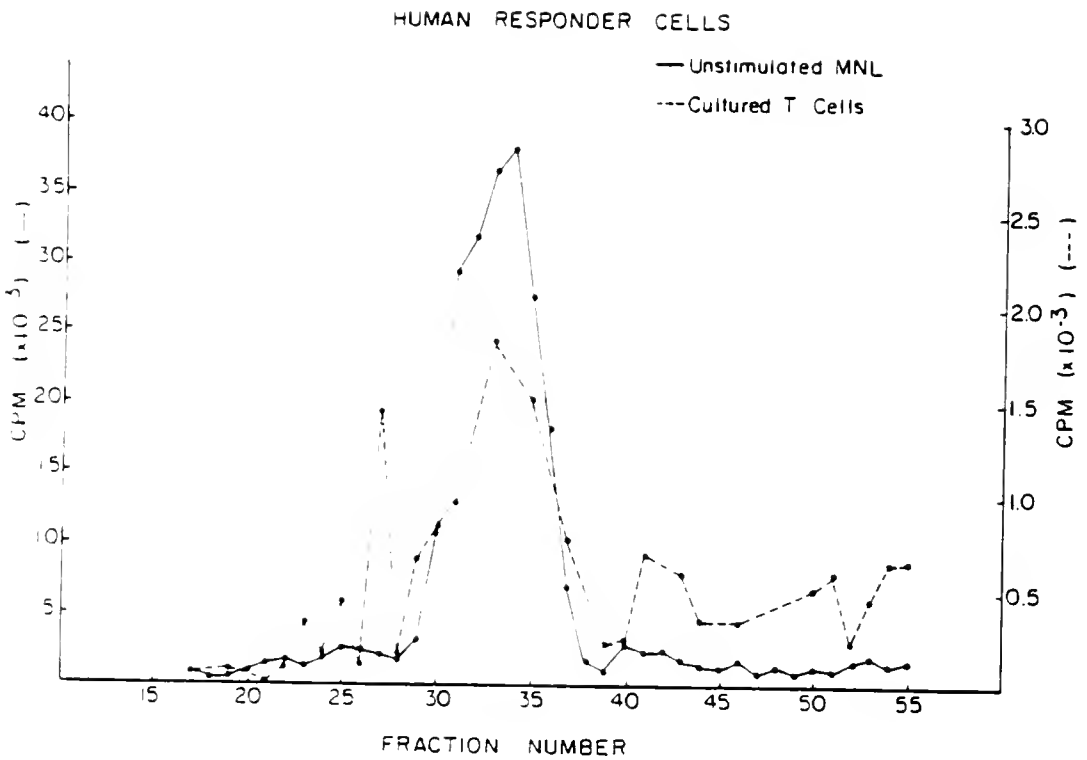

HUMAN RESPONDER CELLS
Unstimulated MNL
Cultured T Cells
CPM (x10-3) (—)
CPM (x10-3) (---)
40
35
30
25
20
15
10
5
3.0
2.5
2.0
1.5
1.0
0.5
15
20
25
30
35
40
45
50
55
FRACTION NUMBER

TABLE 11

COSTIMULATOR ACTIVITY OF MLC AND PLT SUPERNATANTS[a]

Supernatant	cpm
Medium	611 ± 149
PHA	1,158 ± 129
MLC sup.	650 ± 88
MLC sup. + PHA	12,210 ± 2,355
PLT sup.	1,934 ± 725
PLT sup. + PHA	14,977 ± 1,868
Mouse LAF[b]	4,773 ± 342
LAF + PHA	9,174 ± 1,794
Mouse TCGF[c]	1,657 (No S.D.)
Mouse TCGF (50%) + PHA	19,594 (No S.D.)

(a) The costimulator assay was performed as described in Materials and Methods except that PHA (.1%) was used instead of ConA.

(b) LAF (lymphocyte activating factor) was obtained from the supernatant of a mouse macrophage cell line, P388D (from Dr. Shiro Shimuzu). The peak of activity of a Sephadex G100 column fractionation was used for the LAF sample.

(c) Mouse TCGF was obtained from ConA activated spleen cells supernatant (2 days) and treated with α-methyl mannoside (from Dr. Shiro Shimuzu).

costimulator activity of the fractions (Figure 30) shows several peaks with the main peak coinciding with the one seen in the MLC-MF assay. When fractions were tested for their direct mitogenic activity on mouse thymocytes, a direct correlation was found between this peak (Figure 31) and the MLC-MF peak (Figure 27). Thus, the fractions which directly stimulated resting cells of both species were closely associated. A comparison of the two activities is shown in Figure 32. An attempt was made to test fractions on a mouse cytotoxic T cell line which was TCGF-dependent for its growth. Two runs of the fractions showed consistently low stimulation of these cells by fractions from the entire column.

Absorption of MLC-MF Activity

A series of experiments were done to determine whether resting cells could absorb MLC-MF activity. All the absorptions were done at 4°C (to prevent any release of factors) for 30 minutes. The graph presented in Figure 33 shows that the resting MNL were very efficient in absorbing MLC-MF. Not only did fresh MNL absorb MLC-MF but also PHA-stimulated MNL, and a human T lymphoblastoid cell line absorbed it too. In contrast, mouse fibrosarcoma cells did not significantly absorb out any activity.

Supernatants absorbed with resting MNL were also tested for their activity on the TCGF-dependent long term human cell line. Table 12 shows the surprising finding that resting cells absorbed out TCGF activity as measured by the lack of stimulation of the long term cell line. These same supernatants also absorbed MLC-MF activity (Table 12) resting cells.

Figure 30. Assay of column fractions for costimulator activity. Column fractions from a Sephadex G-100 separated MLC supernatant were individually tested in the mouse costimulator assay. All fractions were tested at 50% concentration. Unfractionated MLC supernatant had 21,356 $\pm$ 1,158 cpm.

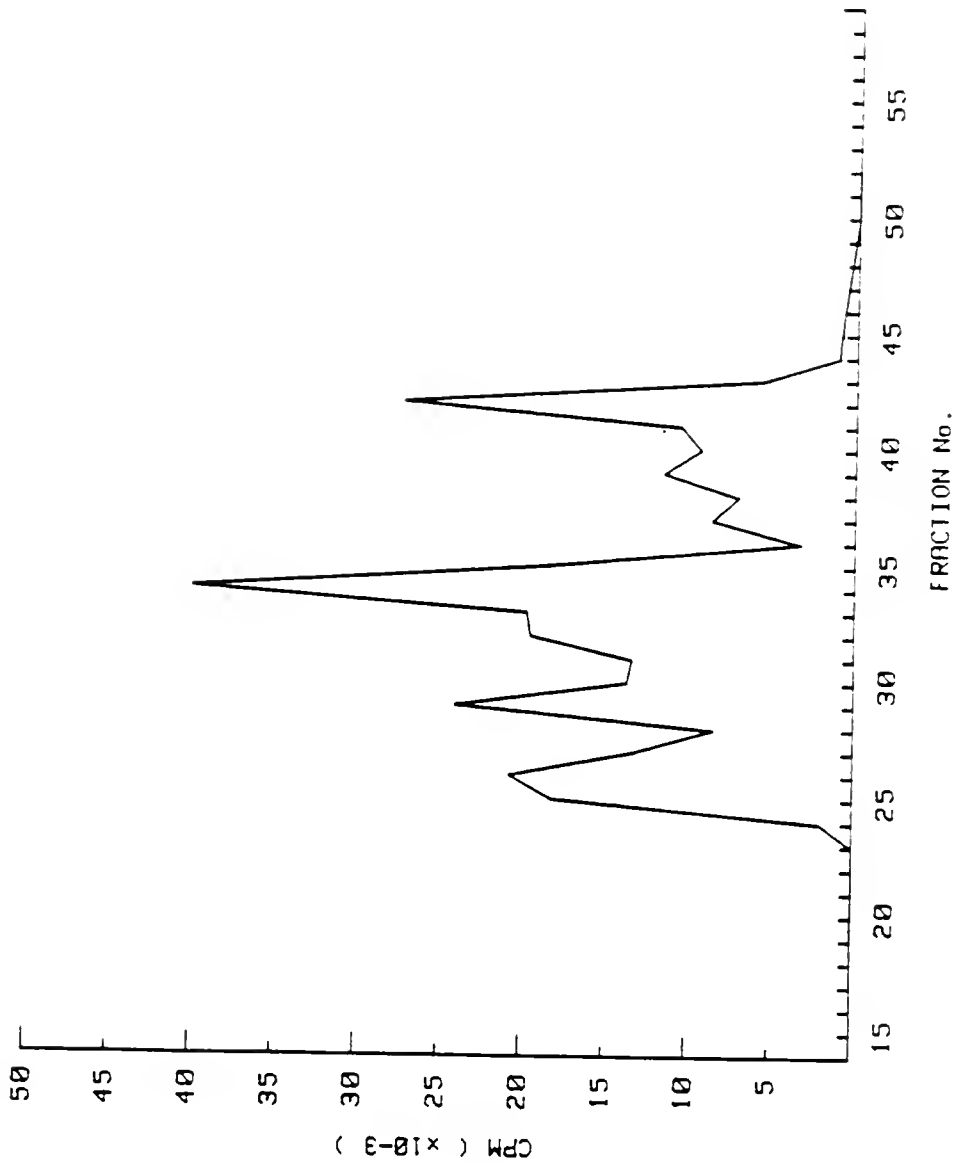

CPM (x10-3)
50
45
40
35
30
25
20
15
10
5
15
20
25
30
35
40
45
50
55
FRACTION No.

Figure 31. Assay of column fractions for mitogenic activity on mouse thymocytes. Column fractions from a Sephadex G-100 separated MLC supernatant were individually tested for their direct mitogenic activity on mouse thymocytes (the same as Figure 30 but in the absence of ConA). Unfractionated MLC supernatant had 2,827 $\pm$ 322 cpm.

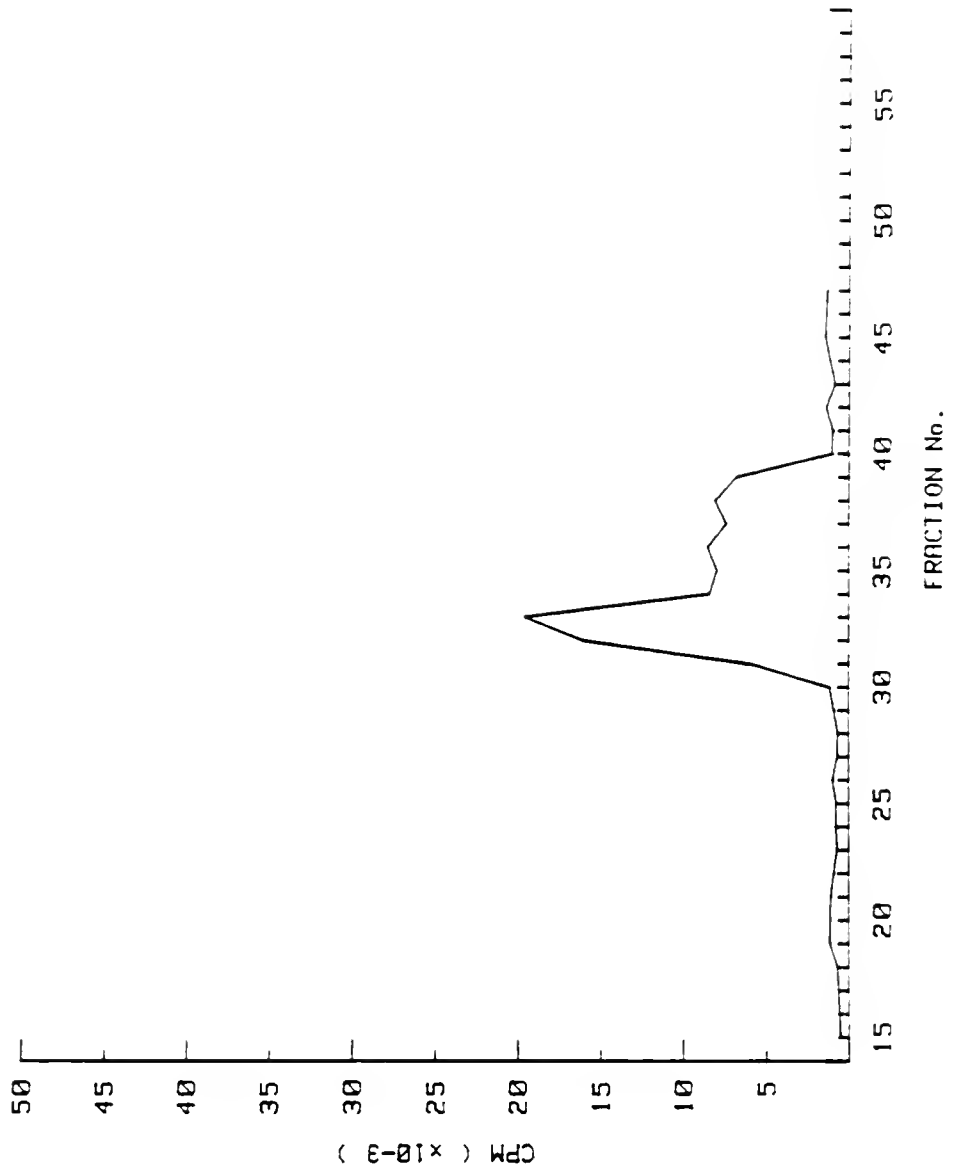

CPM (x10-3)
50
45
40
35
30
25
20
15
10
5
15
20
25
30
35
40
45
50
55
FRACTION No.

Figure 32. A comparison of Figures 30 and 31.

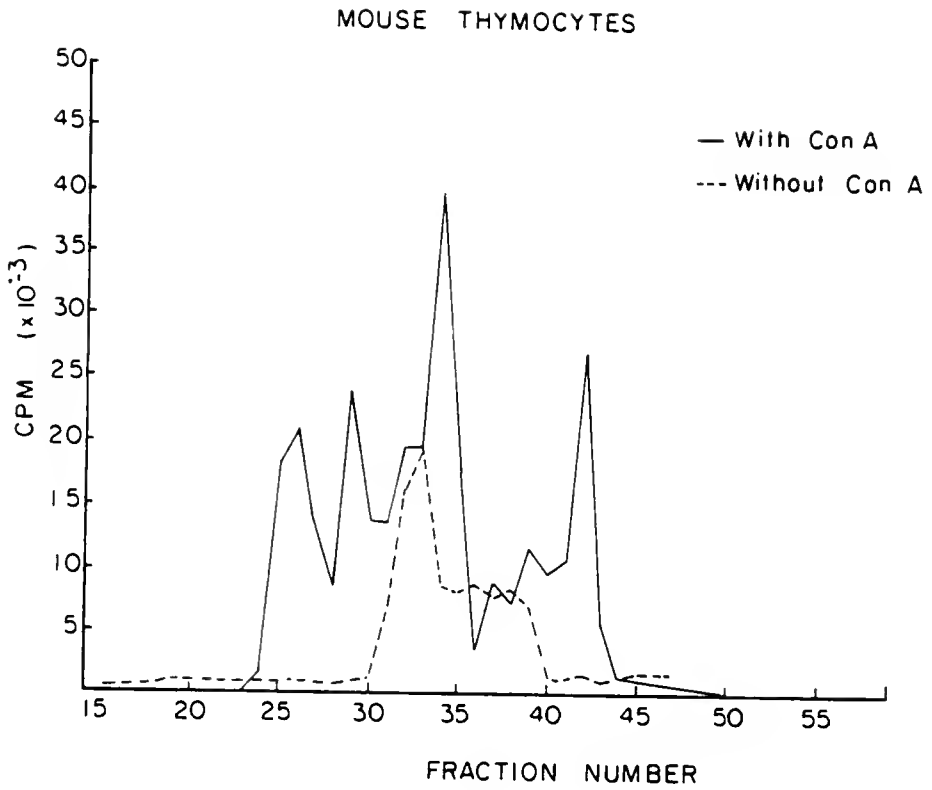

MOUSE THYMOCYTES
— With Con A
--- Without Con A
CPM (x10⁻³)
50
45
40
35
30
25
20
15
10
5
15
20
25
30
35
40
45
50
55
FRACTION NUMBER

Figure 33. Absorption of MLC supernatants with 4 cell populations. The concentrations shown on the x axis are the number of cells per ml of MLC supernatant used to absorb. All the absorptions were done at 4°C for 30 minutes as described in Materials and Methods. The line designations are: M = M3, a mouse fibrosarcoma; T = a human T lymphoblastoid cell line; O = normal human, resting MNL; and P = PHA-stimulated human MNL. The absorbed supernatants were assayed here for their MLC-MF activity (on resting MNL, in the standard assay). The MLC-MF activity of unabsorbed supernatant was 16,091 ± 3,666 cpm. Each point represents the mean of triplicate values.

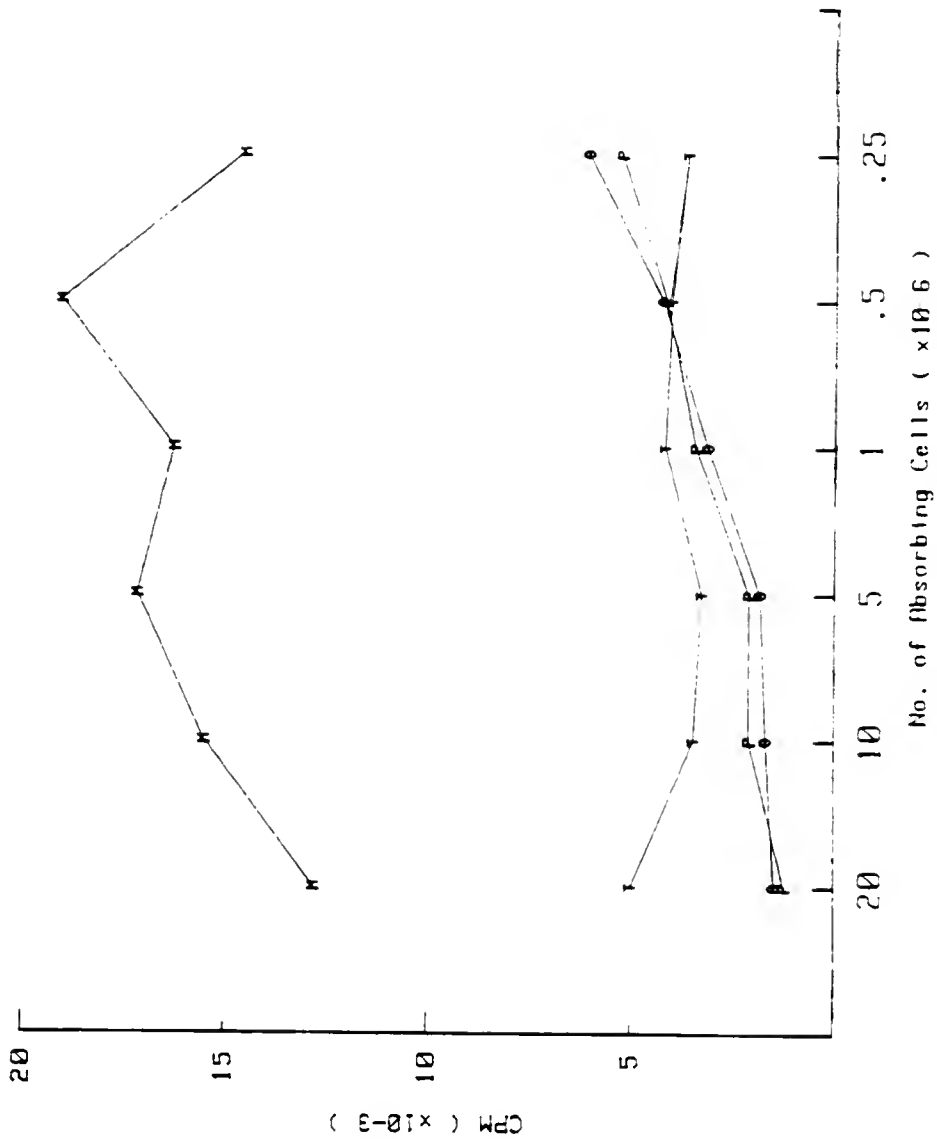

CPM (x10-3)
20
15
10
5
20
10
5
1
.5
.25
No. of Absorbing Cells (x10 6)

TABLE 12

ACTIVITY OF MNL-ABSORBED MLC-MF

Supernatant No.	No.of Resting MNL/ml Used for Absorption	cpm[a] Activity on Resting MNL[b]	cpm[a] Activity on Long Term Line[c]
1	0	36,139 ± 6,892	8,387 ± 1,244
2	20×10^6	10,115 ± 918	3,593 ± 1,528
3	1×10^6	13,547 ± 583	3,252 ± 695
4	$.25 \times 10^6$	11,185 ± 2,320	3,604 ± 1,375

(a) Counts per minute (means of triplicate cultures) ± standard deviation

(b) All supernatants were tested on unstimulated MNL (MLC-MF assay) as described in Materials and Methods.

(c) All supernatants were tested for TCGF levels on the CTCl cell line (TCGF assay) as described in Materials and Methods.

Thus data from the column indicate that costimulator and TCGF (activity on human long term line) activities were found in the same fractions as MLC-MF indicating a close association between these assays. The finding that resting MNL absorbed TCGF activity also supports the notion that MLC-MF and human TCGF are associated. This is in contrast to the mouse system where resting lymphocytes have not been shown to absorb out or respond to TCGF (155). The various possibilities of the association between MLC-MF and human TCGF will be analyzed in the Discussion.

DISCUSSION

I. The Stimulator Cell in MLC

Mixed lymphocyte cultures are thought to be an *in vitro* correlate of the process which occurs *in vivo* during allogeneic graft rejection. The proliferative reaction of these lymphocytes to each other in culture and *in vivo* is determined primarily by differences at the D locus of the HLA complex. The common theory is that a specific clone of helper T cells recognizes the "foreign" D determinants, proliferates (DNA synthesis is what is measured *in vitro* at 6 days), and at the same time provides a helper effect for the maturation of B cells and specific cytotoxic T cells. The entire process, as described above, is dependent on HLA-D antigen being present on the stimulator cell. Thus, it would be expected in primary (and secondary) alloantigen stimulation that cells which share HLA-D determinants would likewise stimulate equally. Historically, this is not supported by data on MLC reactions within families with MLCs in one HLA-D antigen (one-haplotype) and two HLA-D antigen (two-haplotype) mismatch situations (95). A lesser reaction would be expected when there is only one D antigen difference as opposed to a two D antigen difference, based on what is believed to occur during the MLC. The results of most clinical labs, however, show that often the one haplotype MLCs have reactions of comparable magnitude as the two haplotype MLCs. When large numbers of MLCs are analyzed, a statistically significant overlap is found between the mean responses of both groups.

The results presented in this dissertation indicate that the lymphocytes of some individuals stimulate in MLCs higher than others to all responders. The differences in stimulatory capacity are clearly not related to HLA-D disparities between stimulators and responders. This is primarily supported by the experiments with cells from HLA-A,B and D identical individuals (nonreactive to each other in MLC) as stimulators in MLC to a panel of responder cells. Using optimal conditions that were established for the human MLC, three different pairs of HLA-identical siblings were used as stimulators to different responder panels (Figures 8,9 and 10). In all three situations, one sibling was found to be a better stimulator than the other.

These results are supported by analysis of the stimulation induced by some individuals who were tested repeatedly in MLC. Clearly, one individual shown in Table 1 was a good stimulator in all the MLCs he was studied, whereas the other stimulator was consistently low. Figure 6 shows the mean of four separate MLC responses by two individuals to a panel of stimulator cells which had been stored frozen before use. Analysis of the responses indicates that a correlation existed between the responses of both individuals to each stimulator cell (Figure 7). Thus, cells which stimulate high for one responder were also good stimulators for the other responder. The presence of good stimulators can best be exemplified in the studies using panels of ten stimulators and a pool of those stimulators. In almost every case, one or more of the single stimulators induced responses of equal or greater magnitude than the pool of 10. This was also seen in the two-person pools (Table 2) where the stimulation induced by one good stimulator (which has a maximum of 2 HLA-D disparities) was comparible to the pools with 3 to 4

antigen differences. These findings give some insight into what value the MLC has clinically, if any. If cells can induce responses in MLC through non-HLA-D mechanisms, then variations of the magnitude of the MLC can not be solely attributed to HLA-D disparity between the responder and stimulator. If indeed this is the case, then this would be one explanation of the inability of MLCs to effectively predict transplant outcome.

The existence of these "high" and "low" stimulator cell populations could be due to differences in a) concentration of HLA-D antigen bearing cells; b) the number of HLA-D molecules per cell; and c) functional status of the stimulator cells that may alter their capacity to stimulate in MLC.

Monocytes (76,77) and B lymphocytes (75) have repeatedly been shown to be very effective stimulators in MLC. However, the concentration of monocytes and B cells was clearly unrelated to the stimulatory ability of the cell populations studied (Figure 7). Stimulator cells having similar numbers of monocytes and B cells behaved as either high or low stimulators. Activated T cells have been reported to develop "Ia-like" antigens capable of stimulating in MLC (79). However, the number of Ia positive T cells in normal individuals is very low and when resting T cells are tested in MLC they are poor stimulators (59,71). Still, it is possible that a cell unidentified by the methods used here and very active in stimulating in MLCs, such as a peripheral blood equivalent of dendritic cells (80) or even null cells (122) could be responsible for the differences in stimulation. This is an area in which further studies would be necessary to evaluate these possibilities. One way in which certain cells may stimulate better than others may be

related to the relative density or number of HLA-D molecules present on the cell surface. It may be that "good" stimulators possess many more D antigens than "poor" stimulators; however, there is currently no evidence to support this hypothesis.

Functional mechanisms affecting stimulation may involve the presence of suppressor cells in cell populations which stimulate poorly. It is known that suppressor cells which are genetically restricted and specific in their ability to suppress can be found in the peripheral blood of some individuals (113). In addition, alloactivated cell cultures have been shown to induce suppressor cells (106,107). This mechanism although plausible, was not substantiated in experiments where co-culturing cells from a high stimulator sibling with cells of the low stimulator did not depress the response to the high stimulator by unrelated cells (data not shown from Figure 8).

Most reports on the stimulator cell show that it must be metabolically active in order to be able to stimulate. Thus irradiated (3) or mitomycin-C treated (4) cells, which are still viable, serve as excellent stimulators in MLC whereas cells damaged by physical or chemical agents do not stimulate. One possibility is that altering of the HLA-D molecules by the latter agents is what is responsible for the lack of stimulation, although definitive proof of this is lacking. Another interpretation could be that the stimulator cell is secreting some substance (possibly mitogenic) which affects the responder cells in a nonspecific manner. Kasakura (134) demonstrated that irradiated cells were still capable of releasing mitogenic factors and data presented here (Table 5) confirm these results. Lafferty has proposed that stimulator cells release a soluble signal which affects the

responder cell following activation (85). Thus another reason that cells treated with physical agents do not stimulate may be their inability to secrete these soluble products.

II. MLC-induced Mitogenic Factor

In an effort to explore the nongenetic mechanism of cells to behave as good or poor stimulators, experiments were initiated to study mitogenic factors generated during the MLC. The induction of mitogenic factors during the MLC is well documented; however, the role that they play in the actual proliferative process is not known.

Utilizing the ability of these MLC supernatants to stimulate resting MNL, it was found early on that significant variations in the levels of these factors could be obtained by adjusting culture conditions in the MLC. As indicated in Table 5, optimal levels and volumes of MLC-MF (mitogenic factor) could be generated in two-day macro MLC tubes when compared to microtiter plates.

The response to MLC-MF by resting MNL appears to follow the same kinetics as the response to stimulator cells in the MLC, with the peak response in both being 5-6 days (Figure 16). MLC-MF containing supernatants were found to also have the ability to stimulate PLT cells (Figure 16). These cells had a peak response at 2-3 days which mimics the secondary response kinetics these cells demonstrate upon specific alloantigen restimulation. The magnitude of the response to MLC-MF with several responders often approached the response seen in the MLC. In general, responses to MLC-MF were approximately 20-30% of the MLC response.

The magnitude of the MLC response, however, appeared to correlate with the levels of MLC-MF present in the supernatant (Figure 13 and 14). Since MLC-MF is a nonspecific mitogen, this implies that a certain percentage of the proliferation observed in the MLC must be derived from nonspecifically recruited clones of cells.

All initial attempts made to boost MLC responses with MLC-MF demonstrated no effect in most cases and an actual lowering of responses in others. Thus, MLC-MF generated in the MLC appears to be sufficient to saturate the system and any exogenous MF added has no effect.

During the course of these experiments it was discovered that a significant proliferative response occurred when responder cells were in the presence of autologous, irradiated cells and MLC-MF (Figure 11). The cell responsible for this helper effect appears to be the monocyte since the effect of MLC-MF was markedly potentiated by autologous, irradiated MNL containing monocytes but not by monocyte-depleted MNL (Figures 19 and 21). Thus, monocytes are important not only in the generation of MF (142) but also in its effects. However, non-adherent MNL were able to respond to MLC-MF, suggesting that monocytes may not be essential for MF activity. Even though the few monocytes remaining in the non-adherent cell populations could still be playing a role, they were not sufficient to enhance the response to MLC-MF (Figures 19 and 21).

The fact that monocytes are involved in the response of unseparated MNL to MLC-MF brought about the possibility that monocytes (or even the lymphocytes) could be responding to the MLC supernatant by making MF not present in the original MLC-MF preparation. This

possibility is not consistent with the results presented because a) concentrations of hydrocortisone that inhibited MF production did not inhibit the mitogenic effect (Table 7) and b) MLC-MF incubated with MNL for 2 days lost rather than gained mitogenic activity (Figure 23). The conclusion from these experiments is that the activity of MLC-MF is due to the presence of an MF and not to the presence of another factor that secondarily induces production of MF by the responder cell population.

The participation of monocytes in the effect of MLC-MF also introduced the possibility that they could be presenting soluble alloantigens released during the original MLC and inducing activation (and expression of receptors for MLC-MF) on the responding lymphocytes. The availability of two individuals, mother and child, in which the mother responded well to the child's cells, but the child responded poorly to the homozygous HLA-DR mother's cells, allowed testing of this possibility. Supernatants from the MLC of the mother responding to the child might contain, in addition to MLC-MF, soluble alloantigens from both mother and child. If these antigens were contributing in the effect of MLC-MF, then this supernatant would stimulate fresh MNL from the mother (who is responding to the child's HLA-D antigens) but not cells from the child (who does respond to his mother's antigens). The results presented in Table 9 show that the MLC-MF from the mother-to-child MLC, while not as potent as other preparations, induced comparable responses on cells from the mother, the child and an unrelated control. In addition, MLC supernatants generated from three normal persons in MLC combinations between each other, showed no preferential

genetic restriction on fresh MNL from these individuals (Figures 24,25, and 26). These results suggest that it is unlikely that soluble alloantigens have a significant influence on the effect of MLC-MF.

Taken together, these data indicate that MF acts directly on responder lymphocytes not requiring the induction of additional mitogenic substances or the presence of alloantigen. The cells which respond to MFs (produced by MLCs, mitogens or soluble antigens) have been identified as being both T (135,136) and B (139,140) lymphocytes. It has been suggested that the cells which respond to MF are different from those that proliferate in the MLC (146). This suggestion was supported in experiments that were performed treating MNL with OKT4 and OKT8 monoclonal antibodies and complement. The treatment of MNL with OKT4 antibodies, which are directed to determinants present on T helper lymphocytes, effectively prevented the ability of these cells to respond in MLC. In contrast the response to MLC-MF was not affected (Table 10). Pretreatment with OKT8 antibodies and complement produced minimal changes in either the MLC or MLC-MF response.

Of the many activities ascribed to murine TCGF or IL2 (reviewed in Introduction), that characteristic which is both unique and most interesting is the lymphokine's action on activated cells and lack of activity on resting cells (155). This concept, based on absorption and other experiments, basically is that IL2 only works on cells which have interacted with lectin, and not on unstimulated cells. The functional characterization of human IL2 is not as clear as that of murine IL2. Although almost all of the characteristics of murine IL2 have also been found in human IL2 systems, there exists one general exception. It has not been equivocally demonstrated, in any reports to date, that human

IL2 is not stimulatory to resting MNL, as it has been well documented in the mouse. As described in the Introduction, there is an extensive list of reports describing the proliferation of unstimulated peripheral blood MNL by supernatants of human cells stimulated with mitogens or alloantigens (133,143). This is not the case in the mouse system. Allogeneic effect factor (147) remains as one of the only truly mitogenic substances for unstimulated mouse lymphocytes.

Thus, a careful evaluation of the relationship of human IL2 and human mitogenic factors is clearly lacking. In one report, mitogenic factor activity has been referred to as IL2 activity, although there is no evidence to confirm this association (143).

Some of the data presented in this dissertation do establish a relationship between human MF and IL2 activities. The first suggestion of this close association came through column chromatography analysis of MLC supernatants. Fractions of the column which contained high MLC-MF levels also had good costimulator activity on murine thymocytes and growth supporting activity for a long term human T cell line (Figures 27,28 and 30). Both of these latter activities have been described for human IL2. The mitogenic activity of these fractions was not limited to human cells, however, as demonstrated by the induction of proliferation of mouse thymocytes (Figure 31).

The results from the absorption experiments produced unexpected findings and also showed a relationship between MF and IL2. Resting MNL, in addition to PHA-stimulated cells and a lymphoblastoid tumor cell line, were able to absorb not only MF activity (Figure 33) but were also capable of removing IL2 as determined by the lack of activity on the long term cell line (Table 12). The efficiency of this binding

by resting MNL was so high that very few resting cells were needed to absorb either of the activities. These results are not in agreement with others in which human activated, but not unstimulated lymphocytes or lymphoid cell lines were able to remove the IL2 activity of PHA supernatants on cultured T cells (161). One possible reason for these discrepancies are the qualitative and quantitative differences that may exist between the two supernatants. Presently, no detailed comparison of PHA and MLC supernatants has been reported.

The inability to separate human IL2 and MF activities in MLC supernatants brings up several possibilities on their relationship to one another. The first is that human IL2, in a situation analogous to murine IL2, is only acting on activated cells. The source of the activated cells in resting cultures can be from an autologous MLC that is occurring in vitro, or from some in vivo activation process. This would imply that no mitogenic factor exists and that the proliferation seen in resting cells is actually a stimulation of activated cells. The possibility that an autologous MLC is occurring in vitro is not supported by the experiments with hydrocortisone. This drug has been described to inhibit autologous MLCs (118,123), and clearly does not affect the response of resting cells to MLC-MF (Table 7). Thus, if activated cells are indeed proliferating, they would probably be activated in vivo. Levels of activated cells would vary from one individual to the next, thus explaining the different types (high to low) of responders observed to MLC-MF. The demonstration that Ia antigens exist on activated T cells (79) may help exploring this possibility, since, although it is an interesting proposal, no evidence for it presently exists.

Another possibility is that MLC-MF and IL2 are separate, but closely related molecules, each with a unique functional activity. This would be supported by the column data, which cannot separate the two activities. However, the absorption studies would argue against this point. If IL2 only worked on activated cells, then the efficient absorption of IL2 by low numbers of resting MNL could not be explained.

The final possibility is that MLC-MF and IL2 activities reside on the same molecule. This is supported by the column data and would explain the ability of resting MNL and of activated MNL to absorb MLC-MF activity. This latest proposal can be directly approached through better biochemical characterization of the MLC-MF and human IL2 molecule.

Should the latter possibility turn out to be substantiated by future data, it would imply that a fundamental difference exists between murine and human IL2, since mouse IL2 does not apparently possess mitogenic activity on resting cells. However, an aspect of this process that should be considered are the responsive states of human cells in comparison to those of the mouse. In human MLC and mitogen stimulated systems, few cells (e.g. 30 X 10^3) are needed to obtain substantial responses. By contrast, mouse assays require a significantly higher percentage of cells (e.g. 250 X 10^3). Thus, even if mouse IL2 did have mitogenic properties, murine cells may not be able to respond to it. Finally, if IL2 and MLC-MF are the same molecule, then the proposed role that IL2 has in the T cell activation process may have to be revised, since lectin interaction with cells may not be a prerequisite for IL2 action in the human system.

In evaluating the role that MLC-MF plays in the nongenetic ability of cells to stimulate, the relationship remains unclear. Clearly,

MLC-MF has the potential of being a significant influence during the course of an MLC and apparently, levels of MLC-MF in the supernatant may be related to the intensity of an MLC reaction. Since it has been demonstrated that irradiation does not affect the release of MLC-MF, both responders and stimulators in the MLC are probably contributing to the final level measured. Finally, in addition to its role as a potent stimulator, the monocyte may also possess the capacity to enhance an MLC by potentiating the response to MLC-MF that is released. Although the numbers of monocytes do not appear related to the stimulatory ability of a cell population, certainly the functional status of this cell may contribute to the variability among individuals to stimulate in the MLC.

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BIOGRAPHICAL SKETCH

Phillip Ruiz, Jr., was born to Amada and Phillip Ruiz on November 7, 1954, in Tampa, Florida. He grew up there and graduated from Jesuit High School in Tampa in 1972. He attended the University of South Florida and received a B.A. degree in microbiology in 1977. During this time he was awarded the President's Fellowship from the American Society for Microbiology and was placed on the Dean's Honor List. In addition, during his undergraduate education he did a student research fellowship for one year at the National Cancer Institute in the laboratory of Dr. Michael Chirigos. In 1977, he entered the graduate program in the Department of Pathology at the University of Florida where he has worked in the lab of Dr. Juan Scornik. At present, he is a candidate for the degree of Doctor of Philosophy. In September of 1981, Phillip will enter the M.D. program at George Washington University in Washington, D.C.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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